Regulation of Tumor Necrosis Factor-alpha-induced Apoptosis via Posttranslational Modifications in a Human Colon Adenocarcinoma Cell Line

by

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ABSTRACTS

Apoptosis, a physiologically regulated cell death, plays critical roles in development and immune system by maintaining tissue homeostasis. The thesis project investigates regulations of apoptosis in a human colon adenocarcinoma cell line, HT-29, exposed to diverse cellular stimuli, focusing on a specific protein as well as global level of proteins.

The first part of the thesis demonstrated S-nitrosation of procaspase-9. S-nitrosation is a novel protein modification to regulate protein-protein interaction or protein activity. This modification has been implied to inactivate caspases. We could visualize S-nitrosation of an initiator caspase, procaspase-9, by enriching low-abundant procaspase-9 with immunoprecipitation and stabilizing S-nitroso-cysteine with biotin labeling. Nitric oxide synthase inhibitors and tumor necrosis factor-α (TNF-α) reduced the S-nitrosation level of procaspase-9, suggesting that S-nitrosation may be regulated by a nitric oxide synthase and denitrosation is likely a mechanism of apoptosis.

The second part of the thesis is to examine survival effects of insulin on cells undergoing TNF-α-induced apoptosis. Insulin decreased the TNF-α-induced cleavage of key apoptotic mediators, caspases, and their substrates as well as apoptosis, in part, depending on phosphatidylinositol-3 kinase (PI-3K)/Akt pathway. One of protective mechanisms by insulin is likely to decrease the TNF-α-induced dissociation of a potent inhibitor of caspases, X-chromosome linked inhibitor of apoptosis protein (XIAP), from procaspase-9 via PI-3K/Akt pathway.

Lack of phosphoproteomics data in HT-29 cells led the third part of the thesis to focus on investigating global level regulation of phosphoproteins during apoptosis. With a phosphoproteomics technology, IMAC/LC/MS/MS, ~200 phosphosites were identified from HT-29 cells, some of which were detected only from insulin-treated cells. Our phosphoproteomics approach also enabled us to detect alteration of both known and unknown phosphorylation states of apoptosis-related proteins at two time points during early apoptosis induced by tumor necrosis factor-α.

Apoptosis is a multi-step and complex process. Results in this thesis shows that more than one mechanism regulate the activation of a key apoptotic mediator so that fine-tuning exists up to the point of commitment to cell death, when significant alterations of posttranslational modifications occur in protein networks.

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ABBREVIATIONS

Apaf-1, apoptotic protease-activating factor-1

BID, the BH3 domain-containing proapoptotic Bcl-2 family protein

ERK, extracellular-regulated kinase

FLIP, FLICE-like inhibitory protein

GSNO, S-nitrosoglutathione

HPLC, high performance liquid chromatography

IFN-γ, interferon-γ

IKK, Iκ-B kinase

IMAC, immobilized metal ion affinity chromatography

JNK1, c-jun N-terminal kinase 1

MAPKK, mitogen-activated protein kinase kinase

MK2, mitogen-activated protein kinase-associated protein kinase 2

MS, mass spectrometry

NMA, N^G-methyl-L-arginine

NOS, nitric oxide synthase

PARP, poly-(ADP-ribose) polymerase

PI-3K, phosphatidylinositol-3 kinase

SNAP, S-nitroso-N-acetyl-penicillamine

TNF-α, tumor necrosis factor-α

XIAP, X-chromosome-linked inhibitor of apoptosis protein

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Chapter 1. REGULATION OF APOPTOSIS VIA POSTTRANSLATIONAL MODIFICATIONS-AN INTRODUCTION

1.1. APOPTOSIS

Since Kerr et al. proposed the term 'apoptosis' for a phenomenon of controlled cell death in 1972 (1, 2), enormous amount of physiological, molecular biological, and biochemical information on apoptosis has been published and in turn applied to medicinal fields. Apoptotic cells demonstrate distinct morphological features, such as cell shrinkage, membrane blebbing, nucleus condensation, cytoskeletal disruption, and fragmentation of the cell into apoptotic bodies without inflammation, whereas necrosis shows cell swelling and lysis by non-specific cell damages (3-6). Apoptosis is a physiolosically essential process in development and immune defense system by removing unnecessary or harmful cells (1, 2). Abnormal regulation of apoptosis, therefore, leads to diverse disease states including carcinogenesis, autoimmune diseases, and neurodegenerative diseases (5). Apoptotic stimuli include tumor necrosis factor family, transforming growth factor-β, loss of matrix attachment, growth factor withdrawal, glucocorticoids, chemotherapeutic agents, UV and gamma radiation, while growth factors, extracellular matrix, estrogen, and tumor promoters inhibit apoptosis (3-5). Exposed to these diverse extracellular and intracellular signals, multicellular organisms maintain homeostasis in tissues between cell proliferation and apoptosis. Molecular mechanisms of fine-tuning between apoptotic and survival signaling networks are important subjects to elucidate biology of cells and to further contribute to medicinal field by characterizing therapeutic targets. This thesis focuses on posttranslational modifications as regulatory mechanisms of both a specific protein and global level of proteins involved in apoptosis.

1.2. KEY APOPTOTIC MEDIATORS, CASPASES, AND APOPTOTIC SIGNALING PATHWAYS

Cysteine-containing aspartate-specific proteases (caspases) are key mediators of apoptosis (7, 8). The protease family was formerly called interleukin-1β converting enzyme (ICE) family based on the function of the first identified member until the term 'caspase' was adopted in 1996 (7). Active site of caspases possesses a cysteine and cleaves the carboxy-termial of aspartic acid residue in their substrates (9). Specificity of caspase substrates is determined by consensus motives of four amino acids with aspartic acid in the carboxyl-terminal. Based on these sequences, caspases are categorized into group I (caspase-1, -4, -5, and 11; WEHD), group II (caspase-2, 3, and 7; DEXD), and group III (caspase-6, 8, 9, and 10; L/VEXD) (10). According to molecular ordering, apoptotic caspases are also categorized into either initiator caspases (e.g., caspase-8, -9) or executioner caspases (e.g., caspase-3, -6, -7). The former group caspases play as upstream mediators of apoptosis and their structure comprises prodomain for protein-protein interaction, large subunit, and small subunit, while the latter group caspases with short prodomain, large subunit, and small subunit, are activated in downstream events (11, 12). In general, initiator caspases are auto-cleaved by their intrinsic activity after increased local concentration via protein-protein interaction, whereas executioner caspases are processed by active upstream caspases. As a result of exposure to apoptotic signals such as death receptor ligands (e.g., tumor necrosis factor-α and Fas ligand) or cellular damaging agents (13, 14) (e.g., UV and gamma radiation, cisplatin, and staurosphorin), procaspases, inactive zymogens under normal conditions, become cleaved

into their active forms (12). For instance, tumor necrosis factor-α (TNF-α) binds to its receptor, resulting in the association of the receptor with an adaptor protein, TNF receptor-associated death domain protein (TRADD), via a death domain (DD), which subsequently binds to Fas-associated death domain protein (FADD) (15). This association forms a death-inducing signaling complex (DISC) (16), in which procaspase-8 binds to the FADD via a death effector domain (DED) (17-20) and becomes activated through homolytic cleavage (21). In type I cells, active caspase-8 directly cleaves downstream procaspase-3 (11, 22, 23), whereas a small amount of caspase-8 activated in type II cells truncates the BH3 domain-containing proapoptotic Bcl-2 family protein (BID). Truncated BID (tBID) may be myristoylated and consequently translocated into mitochondrial membrane (24). The translocation of tBID induces the release of cytochrome *c* into cytosol (25-29), which also occurs through cellular damaging agents (13, 14). In the presence of cytochrome *c* and dATP (30, 31), apoptotic protease-activating factor-1 (Apaf-1) binds to procaspase-9 via a caspase activation recruitment domain (CARD) (32), forming an apoptosome (30, 31, 33-36), in which procaspase-9 becomes activated. Cleaved caspase-9 processes other downstream procaspases such as procaspase-3 (37-40), which further cleaves downstream substrates such as poly-(ADP-ribose) polymerase (PARP) (41), leading to apoptotic changes (42-48). Given that caspases are key apoptotic mediators, their regulation via fine-tuning such as posttranslational modifications or protein-protein interactions will be informative in elucidating mechanisms of cellular balance. Part of this thesis, therefore, focuses on the investigation of mechanisms to regulate activation of a key apoptotic component, procaspase-9.

1.3. ROLE OF NITRIC OXIDE IN APOPTOSIS

Nitric oxide, a highly diffusible molecule, functions as a signaling molecule or a cellular stress in forms of reactive nitrogen species after reactions with oxygen or other radicals (49). Role of nitric oxide in apoptosis has been controversial and multi-faceted. Thus, depending on not only cell types but also concentration and duration of nitric oxide produced, nitric oxide can function as either a pro- or anti-apoptotic factor (49-51). The general consensus is that physiologically normal levels of nitric oxide protect cells, whereas abnormal production of nitric oxide results in pathological consequence, namely, either apoptosis or necrosis. In this thesis, only anti-apoptotic role of nitric oxide will be discussed. Nitric oxide has been reported to exert its anti-apoptotic functions via the activation of guanylyl cyclase and consequent increase of cGMP levels (52), decrease of cytochrome *c* release by suppressing mitochondrial permeability transition pore (53), or increased anti-apoptotic gene expression, particularly, heat shock protein 70 (54). Recently, a novel modification, S-nitrosation (55, 56), by nitric oxide-mediated signals has been identified among a number of proteins, including receptors (57-59), kinases (60), G-proteins (61-63), redox regulatory proteins (64), transcription factors (65-67), and extracellular matrix proteins (68), as a regulatory mechanism in cell signaling pathways including the apoptotic process. Outcome of S-nitrosation varies among proteins, altering activity or protein-protein interaction (56). S-nitrosation had been only suggested using nitric oxide donors, nitric oxide synthase inhibitors, and/or reducing agents rather than directly identified due to lack of techniques. Then, Jaffrey et al. (69) demonstrated that the modification exists physiologically and is regulated by neuronal

nitric oxide synthase with a method to stabilize nitroso-cysteine moieties by labeling. There are still major questions to be answered in S-nitrosation research; specificity of the modification along with characterization of consensus motif, identification of intracellular nitrosating molecules, and elucidation of denitrosating mechanism (55, 70, 71).

1.4. ROLE OF POSTTRANSLATIONAL MODIFICATIONS IN THE ACTIVATION OF CASPASES I. S-NITROSATION

As described above, caspases are activated via cleavage, which has been used as an apoptotic indicator. On the other hand, posttranslational modifications such as phosphorylation and nitrosation have been implied to inactivate caspases, which motivated us to identify the modifications in this thesis project.

S-nitrosation of caspases has also been suggested to decrease their activity or cleavage in diverse cell types treated with nitric oxide donors or nitric oxide synthase inhibitors, although these studies did not directly characterize S-nitrosation (72-80). On the other hand, S-nitrosation of recombinant active caspase-3 treated with a nitric oxide donor, S-nitroso-N-acetyl-penicillamine (SNAP), was identified by electrospray ionization mass spectrometry (ESI-MS) (81). This report, however, raises the issue of relevance to endogenous caspases in the cells and did not localize the modification site. Then, nitrosation of the active-site cysteine in endogenous procaspase-3 and its denitrosation through Fas signaling were observed in various immune cells using photolysis chemiluminescence (82). Also, the relation of S-nitrosation to cellular

localization of procaspase-3 was addressed (83). They observed that larger fraction of procaspase-3 is nitrosated in mitochondria, but the modification did not affect cellular localization. These reports applied more direct methods to endogenous procaspase-3 compared to previous literature. One concern is that they used the whole immunoprecipitates of procaspases and, therefore, it is possible that S-nitrosation could be detected also from other proteins precipitated with procaspase-3. Focusing on an upstream apoptotic protease, procaspase-9, results in Chapter 2 demonstrate S-nitrosation of procaspase-9 and how this modification is regulated during apoptosis.

1.5. PROTECTIVE MECHANISMS OF SURVIVAL FACTORS

Survival factors, such as insulin, epidermal growth factor, platelet derived growth factor, granulocyte-colony stimulating factor, hepatocyte growth factor, insulin-like growth factor, rescue cells from apoptosis (4, 84) induced by death receptor ligands (85) or DNA damaging agents (86). Phosphatidylinositol-3 kinase (PI-3K)/Akt (85, 87-91), extracellular signal-regulated kinase (ERK) (87, 92), protein kinase A (PKA) (93-96), protein kinase C (α, β, ι, ε, ζ) (97-99), focal adhesion kinase (FAK) (100), and NF-κB (101-104) are known to inhibit apoptosis. Also, anti-apoptotic molecules, regulated by these kinases and thereby regulating pro-apoptotic proteins, have been studied to elucidate protective mechanisms by survival factors. Anti-apoptotic bcl-2 family members (e.g., Bcl-2 and Bcl-XL) inhibit apoptosis by blocking release of cytochrome *c* (105). FLICE-like inhibitory protein (FLIP), a dominant negative form of procaspase-8, has been suggested to inhibit the activation of procaspase-8 by playing the role of its

competitor (106-108). X-chromosome-linked inhibitor of apoptosis protein (XIAP), a multi-functional protein involved in cell cycle regulation, protein ubiquitination, and receptor mediated signaling (109), is known as the most potent endogenous inhibitor of cleaved caspase-3, -7, and -9 (110). In cell-free experiments, recombinant XIAP bound to and inactivated the cleaved forms of caspase-3, -7, and -9 (33, 111-115). Likewise, survival stimuli tightly control the activation of caspases via anti-apoptotic proteins activated in diverse kinase pathways.

Insulin was chosen as a survival signal in this thesis project since preliminary experiment in the Sorger Laboratory at MIT showed that insulin has stronger protective effect against an apoptotic agent, tumor necrosis factor-α, than other growth factors used. Insulin receptor is a heterodimer consisting of two α-subunits and two β-subunits linked via disulfide bonds (116). Upon stimulation, the receptor, a tyrosine kinase, is autophosphorylated (117) and in turn activates other proteins such as insulin receptor substrate-1 (IRS-1) (118), which interacts with p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI-3K) (119). Phosphatidylinositol (3,4,5) P_3 and (3,4) P_2 phosphorylated by PI-3K bind to the plekstrin homology domain of Akt (120), which is now located near membrane to be phosphorylated by phosphatidylinositol (3,4,5) P_3-dependent kinase-1 (PDK-1) (121). Akt is further phosphorylated by another kinase, possibly, mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP kinase-2) (122). A variety of Akt substrates have been identified (123-128), elucidating its roles in cell cycle regulation, glycogen synthesis, protein synthesis, cell proliferation, and anti-apoptosis (129, 130).

1.6. ROLE OF POSTTRANSLATIONAL MODIFICATIONS IN THE ACTIVATION OF CASPASES II. PHOSPHORYLATION

Faleiro et al. (131) suggested multiple species of caspases. They showed that two dimensional gel electrophoresis separation of active caspases, isolated from cell extracts using biotin-labeled caspase-specific inhibitor, resulted in alteration of protein spots among different cells. These multiple species may represent not only modified forms but also different caspases considering that the inhibitor used for isolation is not highly specific for a caspase. Phosphorylation of caspases was first suggested by Martins et al. (132). Treatment of HL-60 human leukemia cell extracts with phosphatase λ followed by affinity isolation of active caspases for subsequent two dimensional gel electrophoresis suggested that phosphorylated forms of caspases may exist. Cardone et al. (126) performed *in vitro* kinase experiment with recombinant Akt and procaspase-9, showing phosphorylation of proform and large subunit of caspase-9. Phosphorylation site was also suggested using peptides synthesized from Akt consensus motif scanning. These experiments adopted recombinant proteins or cell-free system, which might be irrelevant to intracellular events. This limitation motivated us to investigate regulatory mechanisms of endogenous procaspase-9. Recently, phosphorylation of endogenous procaspase-9 induced by mitogen-activated protein kinase kinase 1/2 (MEK 1/2) was identified in HeLa cells using phospho-specific antibody (133). While Akt has been suggested to phosphorylate a serine residue of procaspase-9 in the former report, MEK was shown to phosphorylate a threonine residue in the latter, suggesting that diverse kinases phosphorylate procaspase-9 at different modification sites for tighter regulation of

apoptosis. In this thesis, the effects of a survival signal on alteration of caspases at the level of its activation and interaction with other related proteins were investigated.

1.7. ROLE OF PHOSPHORYLATION IN APOPTOSIS

Although it has been known that phosphorylation is involved in regulation of apoptosis, it is difficult to obtain one clear conclusion from literature, which suggests the complexity of phosphorylation events during apoptosis. First notion of phosphorylation being involved in apoptosis was that cells undergo apoptosis without new protein synthesis after treatment with phosphatase inhibitors or kinase inhibitors (134, 135). However, diversity of both kinases and phosphatases, in combination with their modulating proteins regulated by various cellular inputs, emphasizes importance of understanding phosphorylation events at the level of individual protein in complex networks of the apoptotic process. For example, some tyrosine kinases, such as v-abl, protect cells from apoptosis (136-138). On the other hand, lack of the tyrosine phosphatase CD45 in B-cells increased apoptosis (139). Also, while protein kinase C (PKC) α, β, ε, and ι are anti-apoptotic (97-99, 140-143), isozymes θ, μ and δ are pro-apoptotic (144-150). Particularly, nuclear translocation of PKC δ during early apoptosis (151) is interesting since alterations in phosphorylation of nuclear proteins may lead to apoptotic features in nucleus. Also, cAMP-dependent protein kinase (PKA) type I is suggested as an anti-apoptotic kinase (93-96) based on its phosphorylation of Bad (152), whereas type II is considered pro-apoptotic (153, 154). Role of Akt and mitogen-activated protein kinase (MAPK) in apoptosis seems consistent. Anti-apoptotic functions

of Akt have been demonstrated by its activity to phosphorylate Bad (123-125), caspase-9 (126), and IKK-α (127, 128). In MAPK family, while p38 MAP kinase and c-Jun N-terminal kinase (JNK) activated by ligation of tumor necrosis factor receptors are pro-apoptotic, ERK is an anti-apoptotic kinase (155-157). However, categorization is only general since apoptosis is a complex process and other cellular stimuli or modulating factors need to be considered. More precisely, it is likely that altered activity of kinases seems to cause apoptosis. As described above, key events during apoptosis induced by tumor necrosis factor-α involves activation of initiator caspases resulting from local concentration via protein-protein interactions (21, 32). Assuming that altered phosphorylation leads to apoptosis through caspases, phosphorylation may modulate activation of caspases via regulation of interactions with their known or unknown interacting partners. Alternatively, altered phosphorylation may trigger caspase-independent or complementary apoptotic pathways (158). Phosphorylation also alters susceptibility of proteins to cleavage (159) and cleavage regulates kinase activity as well (160). Apoptotic cells demonstrate cytoskeletal changes. Cytoskeletal components are, accordingly, potential candidates regulated by phosphorylation during apoptosis (161). For instance, vimentin is known to be hyperphosphorylated in response to apoptotic agents (162, 163). Tumor necrosis factor and okadaic acid increased the phosphorylation level of hsp-27 (158, 164) and nucleolin (158). In addition to structural proteins, other unknown phosphoproteins, whose alterations result in apoptotic changes, may be regulated during apoptosis. Presently, phosphoproteomics information of HT-29 cells at the global level is not available. In this thesis project, phosphoproteomics approach was adopted to monitor phosphorylation states of various proteins during early apoptosis.

1.8. PROTEOMICS APPROACHES TO IDENTIFY PHOSPHOPROTEINS

Tremendous progress in the development of mass spectrometry instrumentation along with completion of genome sequencing and efforts in the Bioinformatics field enabled the present proteomics research. Gygi et al. (165) reported that expression level of proteins cannot be predicted from mRNA expression, emphasizing complementary information from proteomics in addition to genomics data. Traditional proteomics adopts one or two-dimensional electrophoresis to separate complex protein mixtures followed by in-gel digestion of each spot for subsequent protein identification by mass spectrometry (166). Challenges in identifying low-abundance proteins from 2D gel led to fractionation of complex mixtures by multiple chromatography (167). These approaches mainly provide information of protein identification. Since functions of proteins are regulated via interactions and modifications, techniques to measure posttranslational modifications, such as phosphorylation, acetylation, glycosylation, cleavage, and to identify their interacting partners have been developed in 'functional proteomics' field (168). Particularly, methods to identify phosphoproteins have been intensively developed to overcome disadvantages of their identification (169, 170). In general, phosphopeptides, especially, from low-abundance signaling proteins, are difficult to detect in a complex mixture containing non-phosphorylated peptides, which raises the issue of limited dynamic range and suppression effects. These challenges led researchers to develop methods to enrich phosphoproteins or phosphopeptides. Enrichment of phosphoproteins using phospho-specific antibodies (166, 171, 172), enrichment of phosphopeptides after chemical modification and subsequent labeling (173, 174), and isolation of

phosphopeptides by precursor ion scanning in mass spectrometry analysis (175-180) have been introduced. In the present thesis, a method to enrich phosphopeptides, Immobilized Metal Ion Affinity Chromatography (IMAC) (181-189), was adopted to identify phosphopeptides and to monitor phosphopeptides potentially regulated during the early apoptotic process. Intrinsic challenges still exist in studying phosphopeptides even with enrichment. Some modified peptides are unstable, resulting in decay during ionization or fragmentation. Also, low sequence coverage may not provide sufficient information to localize phosphorylation sites. In addition to identification, quantitation of peptides in a complex mixture presents another difficulty. Our results and challenges will be discussed in Chapter 4.

1.9. HT-29 CELL LINE

HT-29 cell line is a human colon epithelial adenocarcinoma cell line established from a Caucasian female (information from American Type Culture Collection; ATCC). This cell line was chosen for DARPA MIT Bio-Info-Micro project as a model system to investigate cell decision processes between survival and death. HT-29 cell line is responsive to both tumor necrosis factor-α and insulin, showing characteristics of both type I and II apoptotic pathways, which means that the cleavage of caspase-8, -9, and -3 was observed.

1.10. RESEARCH GOALS

1.10.1. Nitric oxide-mediated signal as an anti-apoptotic factor to regulate the activation of an upstream caspase, caspase-9, was investigated. The effects of chemical reagents altering cellular level of nitric oxide on both apoptosis and the activation of caspases were examined. A novel protein modification, S-nitrosation, in procaspase-9 was visualized and its regulation in responses to diverse cellular stimuli was demonstrated.

1.10.2. In order to understand part of cell decision processes between death and survival, the effect of a survival agent, insulin, on an indicator of apoptosis, cleavage of caspases, was investigated. Also, regulatory mechanisms by insulin were examined focusing on interaction of an initiator, procaspase-9, and an anti-apoptotic molecule, XIAP.

1.10.3. Phosphorylation regulates functions and interactions of proteins, modulating cellular signaling networks. During apoptosis, a programmed cell death, both phosphorylation and dephosphorylation occur in signaling pathways, leading to one outcome, dismantling cells. Focusing on early apoptosis, phosphorylation states of proteins were detected at global level using phosphoproteomics techniques.

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Chapter 2. S-NITROSATION REGULATES THE ACTIVATION OF ENDOGENOUS PROCASPASE-9 IN HT-29 CELLS

2.0. ABSTRACT

Nitric oxide-mediated signals have been suggested to regulate the activity of caspases negatively, yet literature has provided little direct evidence. We show in this thesis that cytokines and nitric oxide synthase (NOS) inhibitors regulate S-nitrosation of an initiator caspase, procaspase-9, in a human colon adenocarcinoma cell line, HT-29. A NOS inhibitor, N^G-methyl-L-arginine (NMA), enhanced the tumor necrosis factor-α (TNF-α)-induced cleavage of procaspase-9, procaspase-3, and poly-(ADP-ribose) polymerase (PARP) as well as the level of apoptosis. NMA, however, did not affect the cleavage of procaspase-8. These results suggest that nitric oxide regulates the cleavage of procaspase-9 and its downstream proteins and, subsequently, apoptosis in HT-29 cells. Labeling S-nitrosated cysteines with a biotin tag enabled us to reveal S-nitrosation of endogenous procaspase-9 that was immunoprecipitated from the HT-29 cell extracts. Furthermore, the treatment with TNF-α as well as NOS inhibitors decreased IFN-γ-induced S-nitrosation in procaspase-9. Results in this thesis show that S-nitrosation of endogenous procaspase-9 occurs in the HT-29 cells under normal conditions and that denitrosation of procaspase-9 enhances its cleavage and consequent apoptosis. We, therefore, suggest that S-nitrosation regulates the activation of endogenous procaspase-9 in HT-29 cells.

2.1. INTRODUCTION

A family of cysteine-containing aspartate-specific proteases (caspases) is a key operator in the apoptotic process (1, 2). Based on molecular ordering, apoptotic caspases are generally categorized into initiator (e.g., caspase-8, -9) and executioner (e.g., caspase-3, -6, -7) caspases (3, 4). Inactive procaspases, existing as latent zymogens under normal conditions, become cleaved into their active forms composed of two large subunits and two small subunits either autocatalytically or via other activated caspases during apoptotic signaling pathways (4). In a death receptor mediated apoptotic pathway, binding of tumor necrosis factor-α (TNF-α) to its cognate receptor triggers a cascade of protein-protein interactions, forming a death inducing signaling complex (DISC) (5). Procaspase-8 becomes recruited to DISC (6-9) and undergoes autocleavage due to its increased local concentration, which is explained by a proximity-induced model (10). In type I cells, a large amount of activated caspase-8 directly cleaves executioner caspases such as caspase-3 (3, 11, 12). On the other hand, a lower level of caspase-8 formed in type II cells leads to further downstream events that mediate the release of cytochrome *c* from mitochondria into cytosol (13-17), which in turn activates another initiator caspase, procaspase-9 (18, 19). In the presence of cytochrome *c* and dATP (20, 21), apoptotic protease-activating factor-1 (Apaf-1) binds to procaspase-9 via a caspase activation recruitment domain (CARD) (22), forming a complex called the apoptosome (20, 21, 23-27). In the apoptosome, caspase-9 is activated to process other downstream caspases including caspase-3 (19, 28-30). Active executioner caspase-3 can further cleave downstream substrates involved in apoptotic changes (31-37), such as poly-(ADP-ribose)

polymerase (PARP) (38). Likewise, the cleavage of procaspases, an irreversible posttranslational modification, has been used as an indicator of apoptosis. On the other hand, reversible modifications, such as phosphorylation (39, 40) or S-nitrosation (41-47), have been implied to inactivate procaspases, although only a few reports on direct identification of these modifications in endogenous procaspases are available. Considering that caspases are key mediators of the apoptotic process, identifying any regulatory modifications of these proteases is crucial to elucidate mechanisms of cellular balancing between survival and death.

The role of nitric oxide in apoptosis has been controversial and multi-faceted. Thus, depending on not only cell types but also concentration and duration of nitric oxide produced, nitric oxide can function as either a pro- or anti-apoptotic factor (48-50). The general consensus is that normal levels of nitric oxide protect cells whereas abnormal production of nitric oxide results in cell death. Also, nitric oxide-induced S-nitrosation of proteins, including receptors (51-53), kinases (54), G-proteins (55-57), redox regulatory proteins (58), transcription factors (59-61), and extracellular matrix proteins (62), has been reported as a regulatory modification in cell signaling pathways (63, 64) including the apoptotic process. S-nitrosation of caspases has also been suggested to decrease their activity or cleavage in diverse cell types treated with nitric oxide donors or nitric oxide synthase inhibitors, although these studies did not directly demonstrate S-nitrosation (41-47, 65, 66). On the other hand, S-nitrosation of recombinant active caspase-3 treated with a nitric oxide donor, S-nitroso-N-acetyl-penicillamine (SNAP), was identified by electrospray ionization mass spectrometry (ESI-MS) (67). This report, however, raises the issue of relevance to endogenous caspases in the cells and did not characterize the

modification site. Nitrosation of the active-site cysteine in endogenous procaspase-3 and its denitrosation through Fas signaling were observed in various immune cells by using photolysis chemiluminescence (68). Also, the relation of S-nitrosation to cellular localization of procaspase-3 was addressed (69). These reports applied more direct methods to endogenous procaspase-3 compared to previous literature. One concern is that they used the whole immunoprecipitates of procaspases and, therefore, it is possible that S-nitrosation could be detected also from other proteins precipitated with procaspase-3.

Combining the separation of the components precipitated with procaspase-9 by molecular weight and a labeling method for S-nitrosated cysteine, we were able to visualize S-nitrosation of endogenous procaspase-9. Furthermore, nitric oxide synthase inhibitors and a death signal decreased S-nitrosation of procaspase-9. These results suggest that denitrosation of endogenous procaspase-9 enhances its cleavage and consequently apoptosis.

2.2. EXPERIMENTAL PROCEDURES

2.2.1. Cell culture and chemical treatment

Human colon epithelial adenocarcinoma cell line, HT-29 (provided from the Peter Sorger lab in the MIT Biology Department), was maintained in McCoy's 5A medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and L-glutamine (Life Technologies) at 37°C under 5% CO_2. Cells were seeded onto culture plates at a density of $5x10^4/cm^2$ and grown for 24 hours. Then, 200 U/ml of interferon-γ (IFN-γ) (Roche Applied Science, Indianapolis, IN) was applied for 24 hours to sensitize the cell line to death signals (70, 71), followed by treatment with 50 ng/ml of tumor necrosis factor-α (TNF-α) (Peprotech, Rocky Hill, NJ) for the indicated hours. Cells were co-treated with TNF-α and nitric oxide inhibitors, N^G-methyl-L-arginine (NMA) (Sigma, St. Louis, MO), 1400w, L-N^5-(1-Iminoethyl)-ornithine, and L-thiocitrulline (CalBiochem, San Diego, CA), while S-nitrosoglutathione (Sigma) was applied for 2 hours before TNF-α treatment. In the Biotin Switch Method, cells were treated with NMA or 1400w for 4 hours following 24 hours of IFN-γ treatment.

2.2.2. Cell death assay

Apoptosis was measured with cell death detection ELISA plus (Roche) according to the manufacturer's instruction. Briefly, cell lysates equivalent to 10^3 cells were incubated with both anti-histone antibody labeled with biotin and anti-DNA antibody conjugated with peroxidase in streptavidin-coated microplates for two hours. Microplate

wells were washed and incubated with substrates for colorimetric measurement at wavelength 405 nm with reference at 490 nm.

2.2.3. Western blotting

Cells lysates were prepared in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.5% Igepal, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, 1 μg/ml bestatin, 1 mM PMSF) and centrifuged at 14,000 g for 30 minutes. Total protein concentration in the supernatant was measured by the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). Equal amounts of proteins were then separated in 15% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA) except 10% gel for poly-(ADP-ribose) polymerase (PARP) by using Bio-Rad mini sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system and transferred onto a PVDF membrane. The membrane was blocked in TBS buffer (20 mM Tris-HCl, 150 mM NaCl) with 0.1% Tween-20 and 5% non-fat milk and then incubated with a primary antibody at 4°C overnight. Anti-mouse caspase-8 antibody, anti-rabbit cleaved caspase-9 antibody, anti-rabbit cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA), anti-mouse PARP antibody, anti-mouse caspase-9 antibody, and anti-mouse Apaf-1 antibody (BD Bioscience, San Jose, CA) were used as primary antibodies. After washing with TBS buffer with 0.1% Tween-20, the membranes were incubated in the blocking buffer with secondary anti-IgG antibody conjugated with horseradish peroxidase (Pierce Biotechnology) for an hour. The membranes were then developed with supersignal West Femto substrate (Pierce Biotechnology).

2.2.4. Immunoprecipitation

50 µl of polyclonal anti-rabbit procaspase-9 antibody (BD bioscience) was immobilized onto 150 µl of Aminolink plus coupling gel beads (Pierce) according to the manufacturer's instruction and stored in the same volume of phosphate buffered saline. For control beads, normal rabbit serum was immobilized under the same conditions. Cell lysates containing 5 mg of total protein were incubated with 20 µl of the immobilized antibody at 4°C overnight. After centrifugation, the supernatant was removed and the beads were washed with TBS buffer containing 0.1% Igepal and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma) extensively. Bound proteins were eluted by 0.1 M of glycine (pH 2.9) and immediately neutralized with ammonium hydroxide.

2.2.5. Detection of S-nitrosation by the Biotin Switch Method

The procedure was performed according to the protocol by Jeffrey and Snyder (72, 73). Briefly, eluates of procaspase-9 immunoprecipitation and rabbit muscle creatine phosphokinase (Sigma) as controls were, respectively, incubated with 20 mM methyl methanethiosulfonate (MMTS) (Sigma) followed by acetone precipitation. Precipitates were centrifuged and resuspended in HENS buffer (25 mM Hepes-NaOH, 0.1 mM EDTA, 0.01 mM neocuproine, and 1% SDS) and then incubated with 1 mM ascorbic acid and 4 mM N-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propionamide (biotin-HPDP) (Pierce Biotechnology) for one hour. Since biotin-HPDP is cleavable under the reducing conditions, prepared samples were loaded onto SDS-PAGE gels without dithiothreitol. Biotinylated samples were then detected by blotting as described above except that 3%

bovine serum albumin was used in the blocking buffer instead of non-fat milk, incubation with primary antibody was omitted due to the biotin labeling, and Neutravidin conjugated with horseradish peroxidase (Pierce Biotechnology) was used instead of secondary antibody. If necessary, each membrane was reprobed with anti-mouse caspase-9 antibody by Western blotting.

2.3. RESULTS

2.3.1. N^G-methyl-L-arginine enhanced the TNF-α-induced apoptosis of HT-29 cells

Treatment with the nitric oxide synthase (NOS) inhibitor, N^G-methyl-L-arginine (NMA), enhanced the level of apoptosis induced by TNF-α at 24 hour point (Figure 2-1A). Also, treatment with NMA alone induced apoptosis of IFN-γ-sensitized cells. The cell death assay detects only the end point of apoptosis, so we did not observe significant increases of cell death at earlier time points. Our results demonstrated that blocking the production of nitric oxide enhanced the effect of a death signal, suggesting in turn that endogenously produced nitric oxide can protect cells. Next, we examined the effects of a nitric oxide donor, S-nitrosoglutathione (GSNO), on apoptosis. We used GSNO since it is a more likely endogenous nitric oxide donor in the cells than other agents. Treatment with GSNO decreased the induction of apoptosis by TNF-α and/or NMA, but did not show statistical significance (Figure 2-1B). The partial inhibitory effect by GSNO could result from its poor permeability into the cells. Another NOS inhibitor, 1400w, also enhanced the TNF-α-induced apoptosis (Figure 2-1C).

2.3.2. Nitric oxide synthase inhibitors enhanced the TNF-α-induced cleavage of caspases.

Figure 2-2 shows that co-treatment with NMA and TNF-α enhanced the cleavage of procaspase-9, -3, and PARP compared to the treatment with TNF-α alone at both 8 and 24 hour time points. However, co-treatment with NMA did not affect the cleavage of procaspase-8. Procaspase-3 and PARP are well-known downstream substrates of

caspase-9 and -3, respectively, meaning that their cleavage can represent the activity of upstream enzymes. Therefore, NMA treatment up-regulated both the cleavage and activity of caspase-9 and -3. Treatment with NMA alone induced the cleavage of procaspase-9 in IFN-γ-sensitized cells (data not shown). These observations suggest that nitric oxide-mediated signals act on the downstream events of caspase-8, which include the activation of procaspase-9. Also, other NOS inhibitors, i.e., 1400w, L-N^5-(1-Iminoethyl)-ornithine, and L-thiocitrulline, enhanced the cleavage of procaspase-9 by TNF-α (data not shown). In addition, pretreatment with S-nitrosoglutathione (GSNO) as a nitric oxide donor decreased the cleavage of procaspase-9, -3, and PARP by TNF-α, again not affecting procaspase-8, and reversed the effect of NMA to enhance the cleavage of procaspase-9, -3, and PARP by TNF-α (data not shown).

2.3.3. The Biotin Switch Method visualized S-nitrosation of procaspase-9.

The results mentioned above indicated that altering the level of cellular nitric oxide by using nitric oxide-related chemicals affected the cleavage of caspases as well as the level of apoptosis. Nitric oxide-mediated signals thus are apparently involved in regulating the cleavage of procaspase-9 and its downstream proteins and consequent apoptosis. Previous reports have implicated S-nitrosation of caspases as a negative regulatory modification (41-47). Since caspase-9 is an upstream caspase and its cleavage was enhanced by the treatment with NOS inhibitors, we focused on visualizing S-nitrosation of endogenous procaspase-9 by using a more direct tool, the Biotin Switch Method (72, 73). Due to the process of biotin labeling and lack of reducing agents in the sample loading buffer for electrophoresis, bands of blotting after the Biotin Switch

Method tend to show more streaking and bending during electrophoresis than usual Western blotting bands. We applied this method to the procaspase-9 immunoprecipitates from the cell extracts of HT-29 cell line pretreated with IFN-γ. IFN-γ is known to up-regulate apoptosis-related genes in our cell line (74), so we used this cytokine to increase the level of low-abundance apoptotic proteins such as procaspase-9. Protein bands representing S-nitrosation appeared at the size of procaspase-9 (47kDa) and higher molecular weight (Figure 2-3A, left), which was shown to be a complex of procaspase-9 formed during the immunoprecipitation process, most likely via disulfide bonds (data not shown). Reprobing the membrane in the left column of Figure 2-3A with caspase-9 antibody showed that bands of S-nitrosation corresponded to those of procaspase-9 (Figure 2-3A, right). Cross reactivity of the antibody was not detected in Western blotting of procaspase-9 following its immunoprecipitation (Figure 2-3B). Also, pretreatment of the procaspase-9 immunoprecipitates with ascorbic acid before the Biotin Switch Method, to reduce S-nitrosated cysteines (72, 73), resulted in weaker or no S-nitrosation than the un-pretreated sample (Figure 2-4A). In addition, no S-nitrosation was visible without the biotin tag (Figure 2-4B). These results indicated that combining immunoprecipitation and the Biotin Switch Method enabled us to visualize S-nitrosation of procaspase-9. There was a possibility that procaspase-9 formed complexes with other proteins and S-nitrosation in higher molecular weight could result also from those proteins. Accordingly, we detected Apaf-1, the only protein known to bind to the proform of caspase-9, forming the apoptosome during the apoptotic process (20, 21, 23-27). Dithiothreitol, which was not present in the sample loading buffer to maintain the cleavable biotin tag intact, was used in lane 2, 4, and 6 to reduce disulfide bonds that may

have occurred during the immunoprecipitation step. Apaf-1 was detected in the original cell extracts. However, as expected, it did not co-immunoprecipitate with procaspase-9 since the cells were not stimulated with apoptotic agents (Figure 2-4C). To avoid the possibility of artificial S-nitrosation by acidified nitrite during elution from the antibody, immunoprecipitates were extensively washed to remove possible nitrite from cell extracts. Also, a control experiment with creatine phosphokinase under the same conditions as that with procaspase-9 showed that elution condition did not affect the level of S-nitrosation. In summary, we could demonstrate S-nitrosation of endogenous procaspase-9 by using immunoprecipitation and the Biotin Switch Method.

2.3.4. Nitric oxide synthase inhibitors and an apoptotic agent decreased S-nitrosation of procaspase-9, which was enhanced by IFN-γ treatment.

Since NOS inhibitors enhanced the TNF-α-induced cleavage of procaspase-9, we separated the effects of NOS inhibitors and TNF-α. Since the Biotin Switch Method is not quantitative, we normalized the density of the S-nitrosation band to that of the procaspase-9 band reprobed with caspase-9 antibody to compare the effects of different chemicals. Compared to untreated cells, treatment with IFN-γ enhanced S-nitrosation of procaspase-9, which was decreased by both NMA and 1400w (Figure 2-5). These results suggest that S-nitrosation of procaspase-9 may be regulated by a NOS induced by IFN-γ. The other interesting observation is that we could visualize S-nitrosation of procaspase-9 in the untreated cells, suggesting that a constitutive level of S-nitrosation may protect cells. In addition, S-nitrosation of procaspase-9 was decreased at the 12-hour point of the incubation with TNF-α (Figure 2-6, BSM/blot). At the same time, we also observed the

cleaved forms of caspase-9, -3, and PARP, whereas the level of Apaf-1, the activator of caspase-9, was not changed (Figure 2-6). Western blotting of procaspase-9 did not reveal a significant change, implying that only a small fraction of procaspase-9 becomes cleaved by TNF-α (Data not shown). These results suggest that TNF-α leads to denitrosation of procaspase-9, promoting its cleavage.

2.4. DISCUSSION

2.4.1. Comparison of our data and previous literature-meaning of our results

TNF-α induces apoptosis through its receptor-mediated signaling pathway, in which upstream caspases, caspase-8 and -9, and a downstream executioner, caspase-3, become activated via cleavage. Considering that these caspases are key mediators of apoptosis, endogenous regulatory mechanisms, such as posttranslational modifications, for their activation must be crucial in maintaining the cellular balance. An anti-apoptotic role of nitric oxide via a posttranslational modification, S-nitrosation, of proteins including caspases has been suggested. Procaspase-3 has been the major target for the detection of endogenous S-nitrosation (68). Also, S-nitrosation was detected in recombinant procaspase-8 added to hepatocyte lysates (43), whereas our results show that the application of a NOS inhibitor, NMA, did not affect the cleavage of endogenous procaspase-8 in HT-29 cells. On the other hand, nitric oxide synthase inhibitors enhanced the TNF-α-induced cleavage of an upstream initiator, procaspase-9, which led us to focus on visualizing its S-nitrosation. We were able to detect S-nitrosation of endogenous procaspase-9 from HT-29 cell extracts by combining immunoprecipitation and labeling endogenously nitrosated cysteine (s). Our data also demonstrated that NOS inhibitors not only enhanced the TNF-α-induced cleavage of procaspase-9, but also decreased S-nitrosation of procaspase-9 based on the Biotin Switch Method. Furthermore, treatment with TNF-α decreased S-nitrosation of procaspase-9, while it induced the cleavage of procaspase-9 and apoptosis. These results demonstrate that TNF-α triggers the cleavage of procaspase-9 via its denitrosation and imply that

denitrosation is part of the regulatory mechanism during the apoptotic process (Figure 2-7). Also, procaspase-9 was S-nitrosated in untreated cells, which suggests that nitric oxide-mediated signals may constitutively protect HT-29 carcinoma cells via S-nitrosation of procaspase-9.

2.4.2. Procaspase-9 might be compartmentalized with a nitric oxide synthase

Since treatment with IFN-γ enhanced S-nitrosation, an important question is which NOS regulated by IFN-γ is involved in increasing S-nitrosation of procaspase-9. IFN-γ-dependent expression of inducible nitric oxide synthase (iNOS) has been observed in diverse cell types (75, 76). Particularly, co-treatment with IL-1α and IFN-γ increased the gene expression of iNOS in HT-29 cells (77). We thus examined the level of iNOS protein as well as the alteration of S-nitrosation of procaspase-9 by these cytokines. iNOS was detected by Western blotting in the cells treated with both IL-1α and IFN-γ for 24 hours, but not with each cytokine alone (data not shown). Also, we could detect neither neuronal nor endothelial nitric oxide synthase, although potential inhibitors of nNOS and eNOS enhanced the cleavage of procaspase-9 by TNF-α (data not shown). Our inability to detect a NOS by Western blotting suggests that the source of nitric oxide may come from a very low level of a NOS compartmentalized with procaspase-9. Such compartmentalization could also explain why S-nitrosoglutathione only partially reversed the effects of NMA on apoptosis (Figure 2-1B) possibly due to the limited accessibility of GSNO to a location of procaspase-9 and its instability due to the reactivity inside of the cells. Recent research has attempted to identify a new NOS isoform in mitochondria (77-82). Whether a NOS exists in mitochondria of HT-29 cells is not known, but it could be

a candidate for S-nitrosation of procaspase-9 in our cell line. The problem of identifying a regulatory NOS isoform is thus also related to the cellular localization of both proform and cleaved form of caspase-9. Mannick and colleagues have attempted to answer this question (69), concluding that S-nitrosation of procaspase-3 did not affect its localization, although a larger fraction of caspase-3 and -9 was S-nitrosated in mitochondria than in cytosol. This conclusion implies that S-nitrosation of procaspase-3 and -9 occurs in the mitochondria. They also reported heme-nitrosation of cytochrome *c* (83), further supporting a role of a mitochondrial NOS in the regulation of apoptosis. Recently, nitric oxide-dependent interaction of procaspase-3 and nitric oxide synthases was also reported, which provides additional confirmation that co-localization of procaspase and nitric oxide synthase is likely and that S-nitrosation is regulatory mechanism of apoptosis {Matsumoto, 2003 133 /id}. Therefore, the identification of a NOS in mitochondria and its relation to S-nitrosation of proteins will provide important information on the regulation of apoptosis. The present limitation is lack of a specific antibody to detect mitochondrial NOS. Antibodies to three pre-existing NOS isoforms have been used to detect mitochondrial NOS (78, 80, 81), but other investigators have failed to confirm these results (82).

2.4.3. Intracellular nitrosating agents and mechanism of denitrosation?

Characterization of intracellular nitrosating agents as well as the mechanism of protein denitrosation remains as important questions. We used NOS inhibitors to block the sources of cellular nitrosating agents. N_2O_3 and nitrosated thiols originating from nitric oxide have been suggested as nitrosating agents (63, 64, 84), but the actual

mechanism is not fully understood. Our results show that TNF-α triggers a signaling pathway that leads to denitrosation. Similarly, another death signal, Fas ligand, reduces S-nitrosation of procaspase-3 measured by photolysis chemiluminescence (68). Therefore, death signals seem to cause denitrosation, but the molecules or steps involved in the mechanism of denitrosation remain to be characterized. The level of Apaf-1, an activator of procaspase-9, was not affected by TNF-α treatment, while procaspase-9 became cleaved. Denitrosation may promote the interaction of procaspase-9 with Apaf-1 via conformational change. Recently, S-nitrosation of thioredoxin, a redox regulator, was reported as an anti-apoptotic mechanism (58). In addition, thioredoxin was shown to prevent monomerization and loss of activity of endothelial NOS induced by exogenous nitric oxide (85). These results suggest that molecules such as thioredoxin could either mediate nitrosation or denitrosation.

2.4.4. Present methodology and its limitation

Due to limitations in techniques, direct identification of S-nitrosation in proteins has been difficult. Currently, the Biotin Switch Method (72, 73) is the best technique to visualize the modification, although this method does not provide quantitative information. S-nitrosation sites in proteins have also been deduced from mutations of cysteine moieties, particularly, active site cysteines. In these types of experiments, protein activity or interactions were measured after the mutations, but the modification sites have not been identified directly. We have been focusing on the direct identification of S-nitrosation sites in endogenous procaspase-9 using mass spectrometry with the challenge of detecting a potential modification site of a peptide in a low-abundance

protein. We have developed an analytical method to enrich S-nitrosated peptides. In this method, we could isolate a synthetic peptide labeled with a cleavable biotin tag by using microcapillary streptavidin column followed by alkylation of free cysteine for mass spectrometry analysis. However, application of this method to exogenously nitrosated proteins demonstrated difficulties most likely due to limit of detection resulting from low-efficient nitrosating reaction and sample loss during the procedure.

2.5. SUMMARY

Using a biotin labeling method combined with immunoprecipitation, we were able to visualize S-nitrosation of endogenous procaspase-9 in the HT-29 cell line. We suggest that nitric oxide-mediated signals induced by IFN-γ protect cells from apoptosis, via S-nitrosation of procaspase-9, which then is removed during the apoptotic process induced by TNF-α as outlined in Figure 2-7. S-nitrosation could be a major negative regulatory mechanism to explain the role of nitric oxide in protecting cells from apoptosis. Also, denitrosation could be one of apoptotic events induced by TNF-α to speed up the cleavage of procaspase-9.

2.6. REFERENCES

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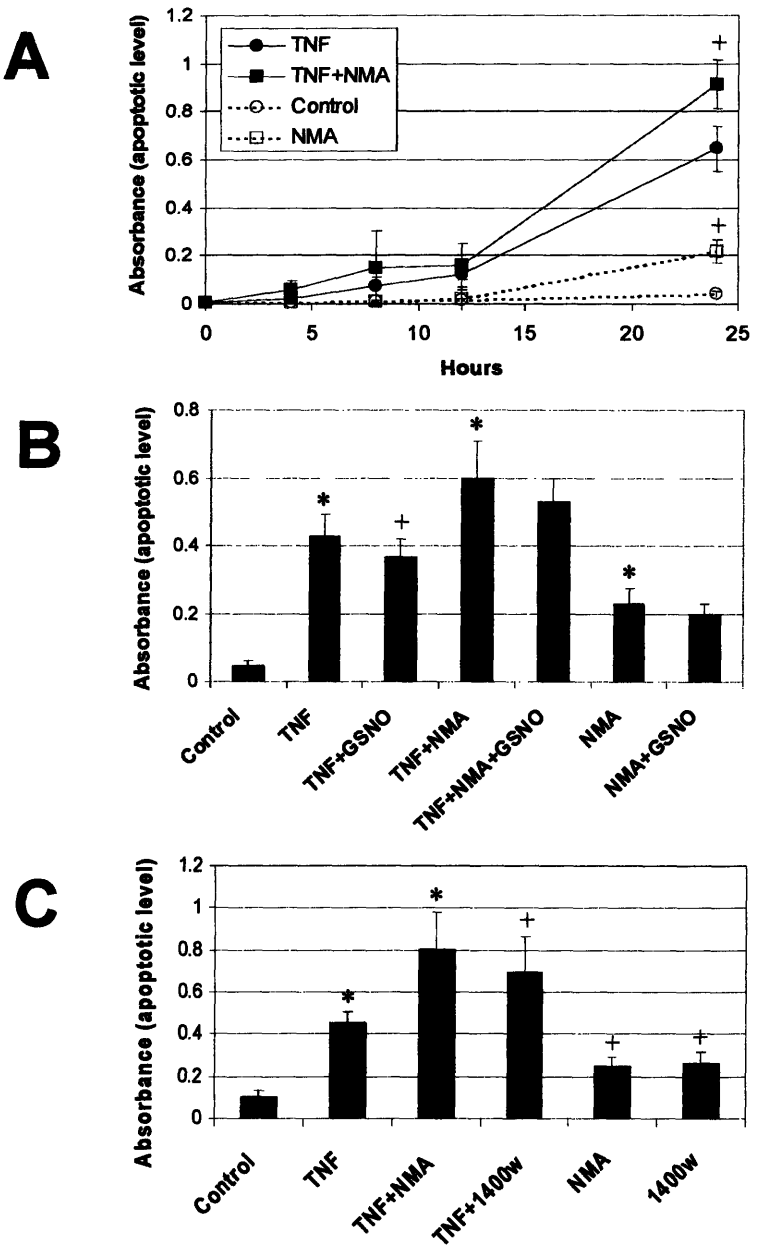

Figure 2-1. The effects of nitric oxide synthase inhibitors on the level of apoptosis induced by TNF-α. The apoptotic level was measured by ELISA assay for cytosolic histone-associated DNA fragment. *Panel A:* Cells were treated with 50 ng/ml of TNF-α and/or 5 mM of NMA for the indicated hours following treatment with 200 U/ml of IFN-γ for 24 hours. *Panel B:* Cells were treated with 50 ng/ml of TNF-α and/or 5 mM of NMA for 24 hours and, in some cases, pretreated with 100 μM of GSNO for two hours following treatment with 200 U/ml of IFN-γ for 24 hours. *Panel C:* Cells were treated with 50 ng/ml of TNF-α and/or 5 mM of NMA or 20μM of 1400w for 24 hours following treatment with 200 U/ml of IFN-γ for 24 hours. In all the graphs, * represents that the value is statistically significant at $p < 0.01$ level. + represents that the value is statistically significant at $p < 0.05$ level. Statistical significance was tested with paired Student's *t* test with $n = 8$.

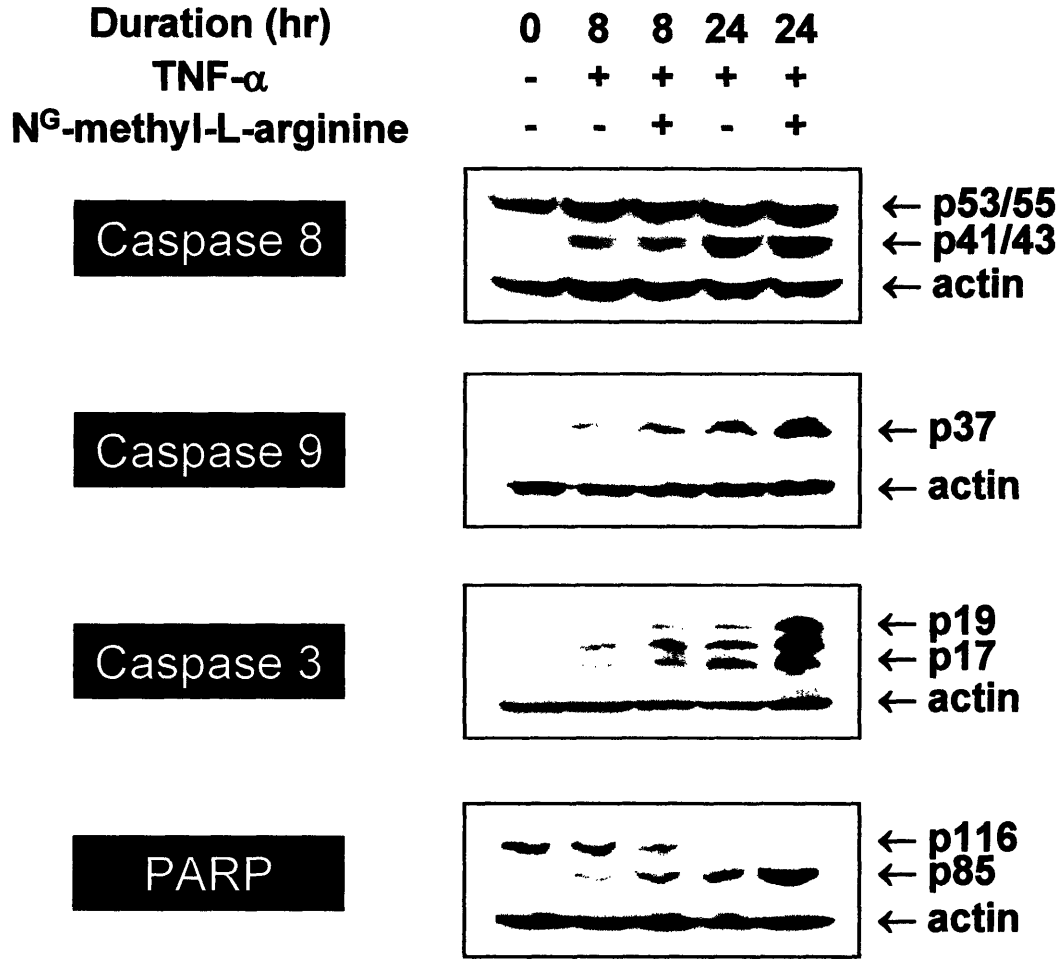

Figure 2-2. The effects of nitric oxide synthase inhibitors on the cleavage of caspases by TNF-α. The intact and/or cleaved form of each protein was detected by Western blotting from the cells incubated with 50 ng/ml of TNF-α and 5 mM of NMA for 8 or 24 hours following treatment with 200 U/ml of IFN-γ for 24 hours. p53/55 of caspase-8 and p116 of PARP are their intact forms. p41/43 of caspase-8, p37 of caspase-9, p17/19 of caspase-3, and p85 of PARP represent their cleaved forms. The figures represent three similar experimental results.

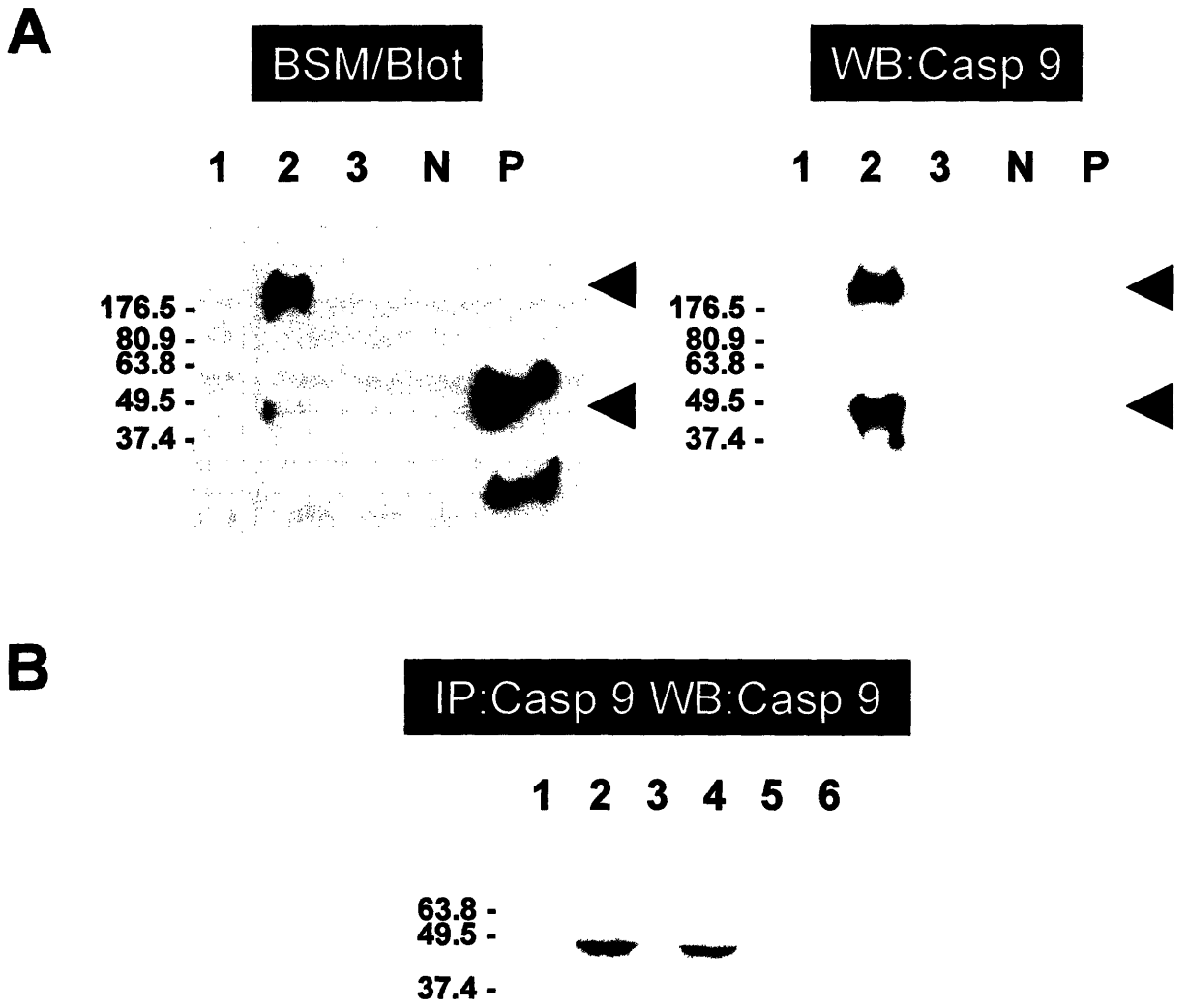

Figure 2-3. The Biotin Switch Method visualizes S-nitrosation of procaspase-9.
Panel A, left: The Biotin Switch Method was applied to the procaspase-9 immunoprecipitates. S-nitrosated proteins labeled with biotin tag were visualized by blotting with Neutravidin conjugated with horseradish peroxidase. lane 1: cell extracts precipitated with control beads, lane 2: cell extracts precipitated with antibody-immobilized beads, lane 3: no cell extracts precipitated with antibody-immobilized beads, lane N: negative control, creatine phosphokinase treated with vehicle and prepared by the Biotin Switch Method, lane P: positive control, creatine phosphokinase treated with 1 mM SNAP and prepared by the Biotin Switch Method. *Panel A, right:* Panel A, left column was reprobed with caspase-9 antibody. *Panel B:* Immunoprecipitation of procaspase-9 followed by Western blotting with caspase-9 antibody. lane 1: cell extracts precipitated with control beads, lane 2: cell extracts precipitated with antibody-immobilized beads, lane 3: no cell extracts precipitated with antibody-immobilized beads, lane 4: supernatant of lane 1, lane 5: supernatant of lane 2, lane 6: supernatant of lane 3.

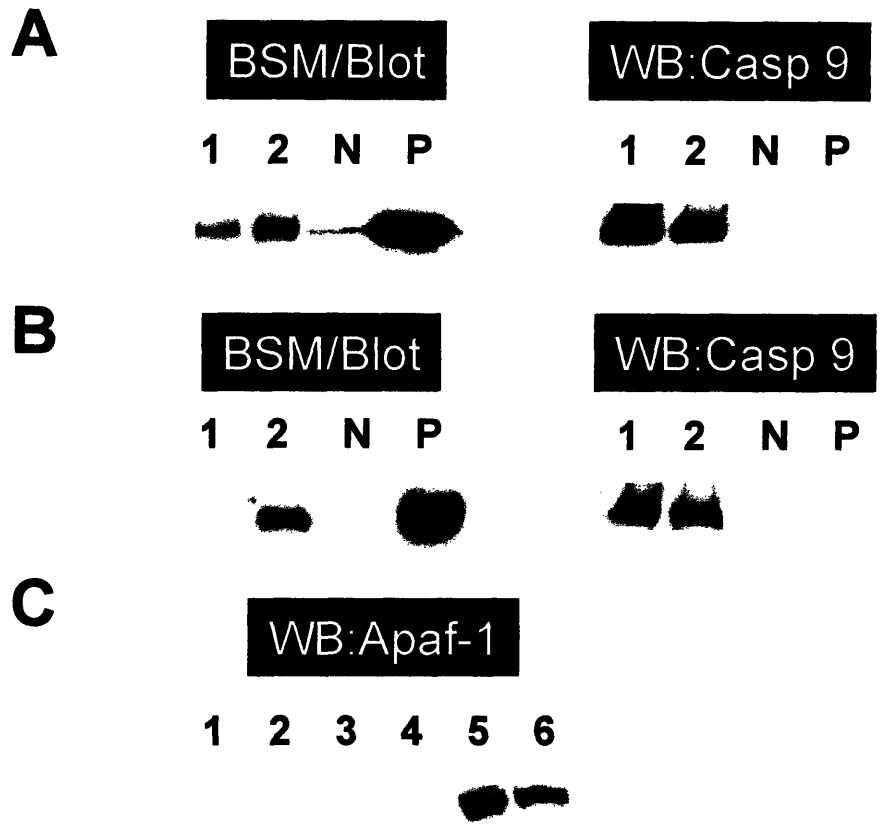

Figure 2-4. The confirmation of S-nitrosation in procaspase-9. *Panel A: The effect of pretreatment with ascorbic acid before the Biotin Switch Method. Left:* lane 1: immunoprecipitates of procaspase-9 pretreated with ascorbic acid before the Biotin Switch Method, lane 2: immunoprecipitates of procaspase-9 followed by the Biotin Switch Method, lane N: negative control, creatine phosphokinase treated with vehicle and prepared by the Biotin Switch Method, lane P: positive control, creatine phosphokinase treated with 1 mM SNAP and prepared by the Biotin Switch Method. *Panel A, right:* The left column was reprobed with caspase-9 antibody. *Panel B: The detection of biotin labeling is specific. Left:* lane 1: immunoprecipitates of procaspase-9 followed by the Biotin Switch Method without biotin-HPDP, lane 2: immunoprecipitates of procaspase-9 followed by the Biotin Switch Method with biotin-HPDP, lane N: negative control, creatine phosphokinase treated with vehicle and prepared by the Biotin Switch Method, lane P: positive control, creatine phosphokinase treated with 1 mM SNAP and prepared by the Biotin Switch Method. *Panel B, right:* The left column was reprobed with caspase-9 antibody. *Panel C: Apaf-1 was not precipitated with procaspase-9.* Apaf-1 was detected by Western blotting. lane 1: immunoprecipitates of procaspase-9 followed by the Biotin Switch Method and loaded onto electrophoresis gel without dithiothreitol, lane 2: immunoprecipitates of procaspase-9 followed by the Biotin Switch Method and loaded onto electrophoresis gel with dithiothreitol, lane 3: immunoprecipitates of procaspase-9 loaded onto electrophoresis gel without dithiothreitol, lane 4: immunoprecipitates of procaspase-9 loaded onto electrophoresis gel with dithiothreitol, lane 5: cell extracts without dithiothreitol, lane 6: cell extracts with dithiothreitol.

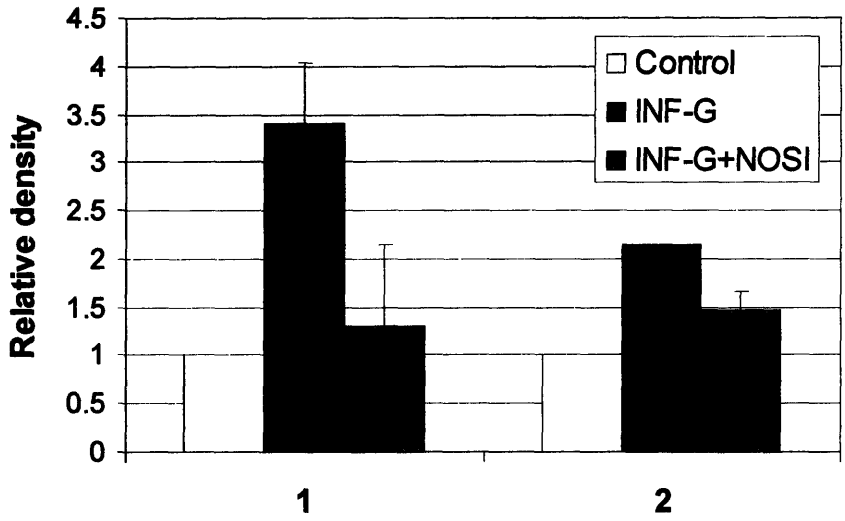

Figure 2-5. The effects of nitric oxide synthase inhibitors on S-nitrosation of procaspase-9. The Biotin Switch Method was applied to immunoprecipitates of procaspase-9 followed by blotting of biotin labeled proteins. Graph shows the relative level of S-nitrosation in procaspase-9. The density of S-nitrosation measured by using Scion Image software (Scion Corporation, Frederick, Maryland) was normalized to that of the procaspase-9 band from Western blotting. Control: no treatment, INF-G: 200 U/ml of IFN-γ, IFN-G+NOSI: 200 U/ml of IFN-γ with 5 mM of NMA (1, the left of the graph) or 20μM of 1400w (2, the right of the graph).

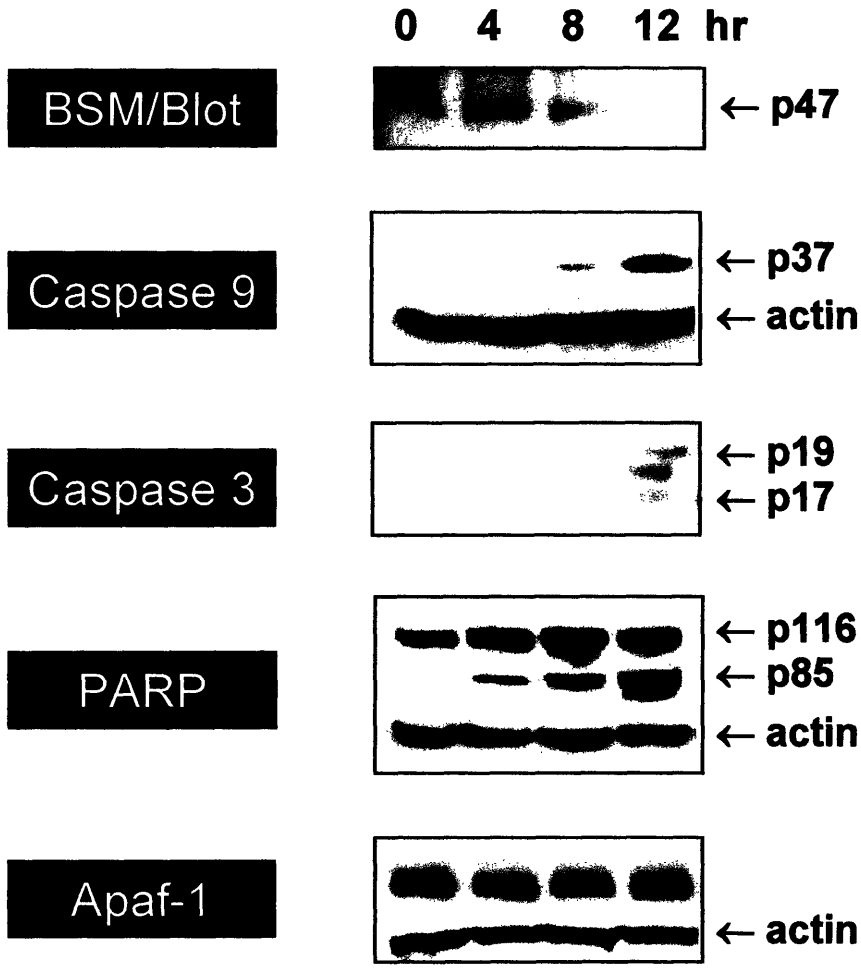

Figure 2-6. The effect of TNF-α on S-nitrosation of procaspase-9. BSM/blot: The Biotin Switch Method was applied to immunoprecipitates of procaspase-9 from HT-29 cells treated with TNF-α for the indicated hours following the treatment with 200 U/ml of IFN-γ. The rest of blots: The intact and/or cleaved forms of each protein were detected by Western blotting from the cells incubated with 50 ng/ml of TNF-α for the indicated hours following the treatment with 200 U/ml of IFN-γ.

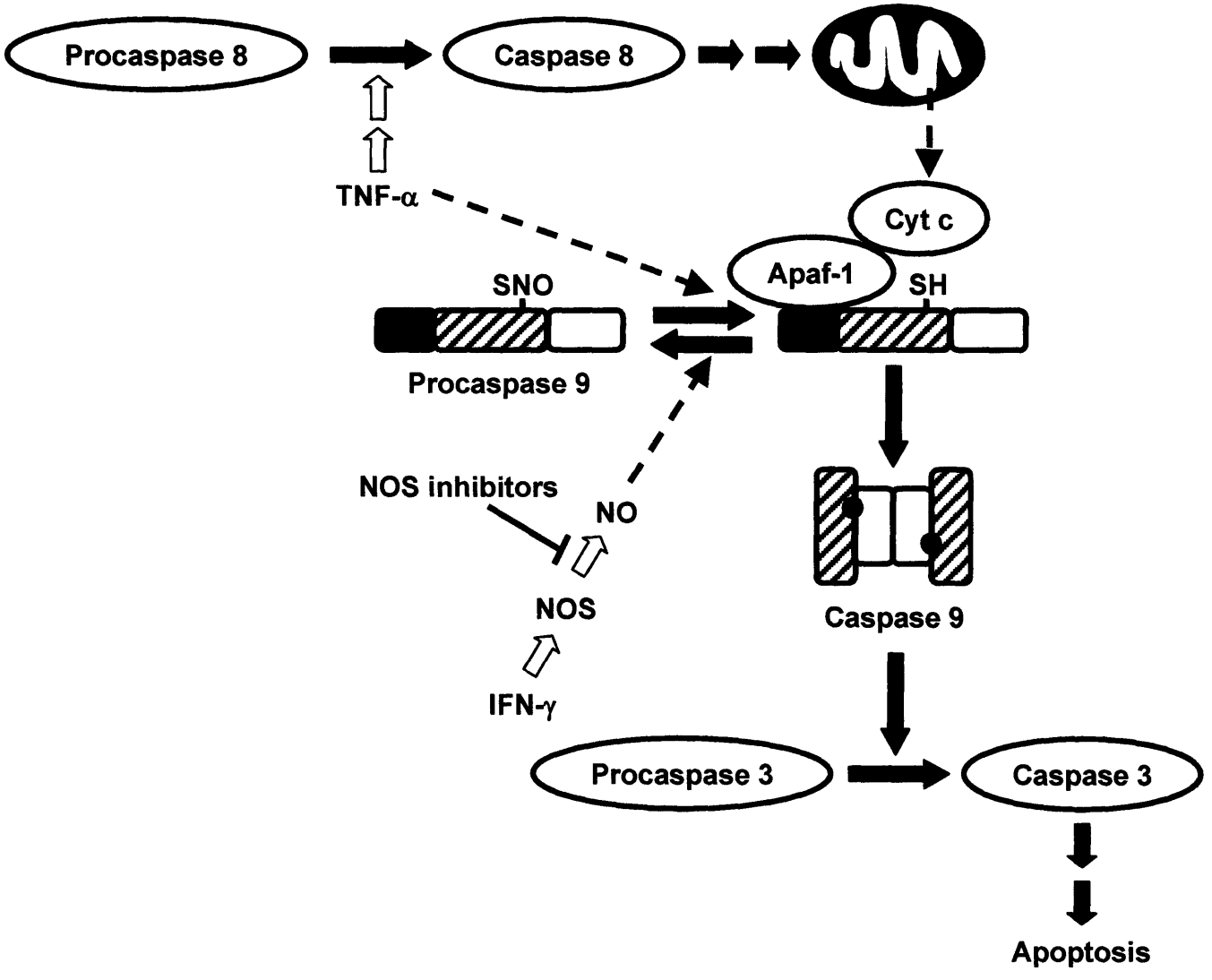

Figure 2-7. A scheme of the regulation of procaspase-9 by cytokines. TNF- , tumor necrosis factor- ; IFN- , interferon- ; Cyt c, cytochrome C; Apaf-1, apoptotic protease-activating factor-1; NOS, nitric-oxide synthase; NO, nitric oxide; SNO, *S*-nitroso; SH, sulfhydryl.

Chapter 3. THE INHIBITORY EFFECTS OF INSULIN ON THE ACTIVATION OF PROCASPASE-9 VIA X-CHROMOSOME LINKED INHIBITOR OF APOPTOSIS PROTEIN

3.0. ABSTRACTS

Insulin significantly reduced the tumor necrosis factor-α (TNF-α)-induced cleavage of procaspase-8, -9, -3, and poly-(ADP-ribose) polymerase when observed up to 24 hours and in a dose-dependent manner. Signaling pathways responsible for the inhibitory effects of insulin were investigated by using protein kinase inhibitors. Both phosphatidylinositol-3 kinase (PI-3K) and mitogen-activated protein kinase kinase (MAPKK) pathways mediate the ability of insulin to decrease the TNF-α-induced cleavage of procaspase-8. In contrast, only the PI-3K inhibitor reversed the effect of insulin on the TNF-α-induced cleavage of procaspase-9. Moreover, insulin decreased the apoptotic level induced by TNF-α, while the PI-3K inhibitor enhanced it. The protein level of Apaf-1, an activator of procaspase-9, remained constant with the application of agents affecting the cleavage of procaspase-9. In examining a known regulator of cleaved caspase-9, X-chromosome-linked inhibitor of apoptosis (XIAP), we observed that TNF-α treatment induced fragmentation of XIAP, which was also enhanced by the PI-3K inhibitor. In addition, XIAP was co-immunoprecipitated with procaspase-9. The treatment with TNF-α reduced the level of XIAP precipitated with procaspase-9, whereas insulin reversed this effect. Moreover, PI-3K and Akt inhibitors, but not mTOR inhibitor, inhibited the effect of insulin on the co-precipitation of procaspase-9 and XIAP. Our data suggest that insulin decreases the TNF-α-induced cleavage of procaspase-9 and subsequent apoptosis by regulating XIAP via the PI-3K/Akt pathway.

3.1. INTRODUCTION

Apoptosis plays an essential physiological role in development and immune defense system by removing unnecessary or harmful cells (1, 2). Cysteine-containing aspartate-specific proteases (caspases) including initiator (e.g., caspase-8, -9) and executioner (e.g., caspase-3, -6, -7) caspases (3, 4) are key mediators of apoptosis (5, 6). Triggered by apoptotic signals such as death receptor ligands or cellular damaging agents (7, 8), procaspases, inactive zymogens under normal conditions, become cleaved into their active forms (4). Binding of a death receptor ligand, tumor necrosis factor-α (TNF-α), to its receptor results in the association of the receptor with an adaptor protein, TNF receptor-associated death domain protein (TRADD), via a death domain (DD), which subsequently binds to Fas-associated death domain protein (FADD) (9). This association forms a death inducing signaling complex (DISC) (10), in which procaspase-8 binds to the FADD via a death effector domain (DED) (11-14) and becomes activated through homolytic cleavage (15). In type I cells, active caspase-8 directly cleaves downstream procaspase-3 (3, 16, 17), whereas a small amount of caspase-8 activated in type II cells truncates the BH3 domain-containing proapoptotic Bcl-2 family protein (BID). Truncated BID (tBID) may be myristoylated and consequently translocated into mitochondrial membrane (18). The translocation of tBID induces the release of cytochrome *c* into cytosol (19-23), which also occurs through cellular damaging agents (7, 8). In the presence of cytochrome *c* and dATP (24, 25), apoptotic protease-activating factor-1 (Apaf-1) binds to procaspase-9 via a caspase activation recruitment domain (CARD) (26), forming an apoptosome (24, 25, 27-30), in which procaspase-9 becomes

activated. Cleaved caspase-9 processes other downstream procaspases such as procaspase-3 (31-34), which further cleaves downstream substrates such as poly-(ADP-ribose) polymerase (PARP) (35), leading to apoptotic changes (36-42). Regulating the cleavage of various caspases by survival factors, therefore, is essential for the cellular balance between survival and death.

Survival factors such as insulin and growth factors rescue cells from apoptosis induced by death receptor ligands (43) or DNA damaging agents (44). Phosphatidylinositol-3 kinase (PI-3K)/Akt (43, 45-49), mitogen-activated protein kinase (MAPK) (45, 50), focal adhesion kinase (FAK) (51), and NF-κB (52-55) become activated to inhibit apoptosis. Also, anti-apoptotic molecules, regulated by these kinases and thereby regulating pro-apoptotic proteins, have been studied to elucidate protective mechanisms by survival factors. For instance, FLICE-like inhibitory protein (FLIP), a dominant negative form of procaspase-8, has been suggested to inhibit the activation of procaspase-8 by playing the role of its competitor (56-58). Also, Akt was reported to phosphorylate the proform and large domain of recombinant caspase-9 in an *in vitro* kinase experiment (59). X-chromosome-linked inhibitor of apoptosis protein (XIAP), a multi-functional protein involved in cell cycle regulation, protein ubiquitination, and receptor mediated signaling (60), is known as the most potent endogenous inhibitor of cleaved caspase-3, -7, and -9 (61). In cell-free experiments, recombinant XIAP bound to and inactivated the cleaved forms of caspase-3, -7, and -9 (27, 62-66). Likewise, it has been suggested that survival stimuli tightly control the activation of caspases via posttranslational modifications or protein-protein interactions.

Assuming that survival factors protect cells from apoptosis by inactivating key apoptotic mediators, caspases, via anti-apoptotic proteins, we examined the effects of a survival factor, insulin, on the cleavage of major procaspases and a substrate, PARP. Also, we defined an insulin kinase pathway (s) responsible for the inhibitory effects of insulin on the cleavage of procaspases. Focusing on caspase-9, we observed that XIAP was co-immunoprecipitated with procaspase-9 and also cleaved following TNF-α treatment. The action of XIAP was, therefore, investigated at the level of interaction with procaspase-9. Our results suggest that insulin decreases the TNF-α-induced cleavage of procaspase-9 and apoptosis by regulating binding of procaspase-9 and XIAP via PI-3K/Akt pathway.

3.2. EXPERIMENTAL PROCEDURE

3.2.1. Cell culture and chemical treatment

Human colon epithelial adenocarcinoma cell line, HT-29 (generously provided by the Peter Sorger lab in the MIT Biology department), was seeded with a density of $5x10^4$/cm in McCoy's 5A medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and L-glutamine (Life Technologies) at 37°C in 5% CO_2 incubator. 200 U/ml of interferon-γ (IFN-γ) (Roche Applied Science, Indianapolis, IN) was applied for 24 hours to sensitize the cells to apoptotic agents (67, 68). Then, 50 ng/ml of tumor necrosis factor-α (TNF-α) (Peprotech, Rocky Hill, NJ) with or without insulin (CalBiochem, San Diego, CA) was applied to the cells for the specified hours. In some experiments, cells were pretreated with protein kinase inhibitors, LY 294002, PD 98059, Rapamycin, Akt inhibitors (CalBiochem), for one hour.

3.2.2. Western blotting

Cell lysates were prepared by three freeze-and-thaw cycles in a lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.5% Igepal, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 μM of okadaic acid, 1 μg/ml leupeptin, 1 μg/ml bestatin, and 1 mM PMSF) followed by centrifugation at 14,000 g for 30 minutes. For cytochrome *c* detection, cytosolic fraction was prepared by incubating cells in a lysis buffer with 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 μM of okadaic acid, 1 μg/ml leupeptin, 1 μg/ml bestatin, and 1

mM PMSF at 4°C for 20 minutes followed by 15 passages through 26 gauge needle. Then, lysates were centrifuged at 14,000 g for 30 minutes followed by the centrifugation of the supernatant at 100,000 g for 30 minutes. 50 or 100 μg of total protein measured by the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL) was separated in 15% Tris-HCl gel (BioRad Laboratories, Hercules, CA) by SDS-PAGE except 10% gel for poly-(ADP-ribose) polymerase (PARP) and transferred onto a PVDF membrane. The membrane was blocked with 5% non-fat milk in Tris buffered saline (TBS) with 0.1% Tween-20 for an hour and then incubated with an indicated primary antibody in the same blocking buffer at 4°C for overnight. Anti-mouse caspase-8 antibody, anti-rabbit cleaved caspase-9 antibody, anti-rabbit cleaved caspase-3 antibody, anti-mouse BID antibody, anti-rabbit XIAP antibody (Cell Signaling Technology, Beverly, MA), anti-mouse cytochrome *c* antibody, anti-mouse PARP antibody, anti-mouse caspase-9 antibody, anti-mouse Apaf-1 antibody, anti-mouse XIAP antibody (BD Bioscience, San Jose, CA), and anti-mouse caspase-9 antibody (Upstate Biotechnology, Waltham, MA) were used as primary antibodies (1:1000 dilution). After washing with TBS with 0.1% Tween-20, the membrane was incubated with secondary anti-IgG antibody conjugated with horseradish peroxidase (1:100,000 dilution) (Pierce Biotechnology) for one hour. Then, blots were developed with supersignal West Femto substrate (Pierce Biotechnology). If necessary, the membrane was reprobed with anti-goat actin antibody (CalBiochem) for normalization.

3.2.3. Apoptosis measurement

Apoptosis levels were measured with cell death detection ELISA plus (Roche) according to the manufacturer's instruction. Briefly, cell lysates equivalent to 10^3 cells were reacted with both anti-histone antibody labeled with biotin and anti-DNA antibody conjugated with peroxidase in streptavidin-coated microplates for two hours. Microplate wells were washed and incubated with substrates for colorimetric measurement at wavelength 405 nm with reference at 490 nm.

3.2.4. Immunoprecipitation

Cell lysates containing 2 mg of protein were incubated with anti-rabbit procaspase-9 antibody (BD bioscience) immobilized onto Aminolink plus coupling gel (Pierce) at 4°C overnight. Beads were washed with TBS containing 0.1% Igepal and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma). Then, bound proteins were eluted with 0.1 M glycine (pH 2.9) for 15 minutes and immediately neutralized with ammonium hydroxide.

3.3. RESULTS

3.3.1. Insulin decreased the TNF-α-induced cleavage of procaspases and their substrates

We investigated whether a survival signal, insulin, affected the cleavage of procaspases and other related proteins in HT-29 cells by using Western blotting. We observed the earliest TNF-α-induced cleavage products of caspase-8 at 4 hour, caspase-9 at 8 hour, caspase-3 at 12 hour, and poly-(ADP-ribose) polymerase (PARP) at 8 hour (Figure 3-1). Due to differences in affinities of each antibody, absolute comparison between proteins at each time point is not possible. However, an overall trend is that upstream procaspases become cleaved before downstream substrates in response to TNF-α treatment. On the other hand, insulin delayed the TNF-α-induced cleavage of all four proteins (Figure 3-1). Also, insulin decreased the TNF-α-induced cleavage of procaspase-8, and -9 and their downstream substrates, BID, procaspase-3, and poly-(ADP-ribose) polymerase (PARP), in a dose-dependent manner (Figure 3-2). The cleavage of the downstream substrates represents the activity of upstream enzymes (e.g., activated caspase-9 cleaves procaspase-3). We also detected release of cytochrome *c* into the cytosol, a downstream event of the caspase-8 activation. The treatment with insulin caused little decrease in the level of cytosolic cytochrome *c*, yet reduced the cleavage of procaspase-9 significantly (Figure 3-2). This result implies that insulin inhibits the cleavage of procaspase-9 via a separate mechanism from the one that affects the cleavage of procaspase-8, an early event of the TNF-α signaling pathway.

3.3.2. PI-3K and MAP kinase pathways mediate the inhibitory effects of insulin

Next, we examined insulin signaling pathways that inhibit the cleavage of procaspases by using protein kinase inhibitors. LY 294002 and PD 98059 are inhibitors of phosphatidylinositol-3 kinase (PI-3K) and mitogen-activated protein kinase kinase (MAPKK), respectively, which are kinases activated by insulin. Both LY 294002 and PD 98059 not only reversed the inhibitory effects of insulin but also enhanced the TNF-α-induced cleavage of procaspase-8. In contrast, only LY 294002 reversed the ability of insulin to inhibit the TNF-α-induced cleavage of procaspase-9 (Figure 3-3). Based on these results, both MAPKK/ERK and PI-3K/Akt pathways activated by insulin reduce the cleavage of procaspase-8, whereas PI-3K/Akt pathway seems to play a major inhibitory role in regulating the cleavage of procaspase-9. Western blotting of their substrates, caspase-3 and PARP, showed corresponding results to those of their upstream proteins. Since both caspase-8 and -9 can cleave procaspase-3, we could not determine whether insulin affects procaspase-3 and PARP directly or via alterations of one or both upstream caspases. In addition, protein kinase inhibitors alone could induce the cleavage of procaspase-8, -9, -3 and PARP, suggesting that an endogenous protective mechanism (s) exists in this cell line.

3.3.3. The effects of insulin and a PI-3K inhibitor on the TNF-α-induced apoptosis and on regulatory proteins of procaspase-9

To confirm the anti-apoptotic role of insulin via the PI-3K/Akt pathway, levels of apoptosis was measured in response to insulin and/or LY 294002 in combination with TNF-α. Insulin rescued cells from TNF-α-induced apoptosis while LY 294002 enhanced

it (Figure 3-4). This result combined with the Western blotting data of caspase-9 suggests that the PI-3K/Akt pathway mediates one of the anti-apoptotic mechanisms activated by insulin via the inhibition of procaspase-9 cleavage. Accordingly, we investigated regulatory proteins of caspase-9. While we could observe that TNF-α cleaved procaspase-9 (Figure 3-2 and 3-3), the treatment with TNF-α for up to 12 hours did not change the protein level of Apaf-1, which is necessary for the activation of procaspase-9 (Figure 3-5A). Also, insulin with or without LY 294002 in combination with TNF-α did not alter the level of Apaf-1 (Figure 3-5B and C). Therefore, we examined another regulatory molecule, XIAP, a potent endogenous inhibitor of cleaved caspase-3 and -9. We observed that the treatment with TNF-α produced an approximately 30kDa fragment of XIAP (Figure 3-6A). In order to obtain complementary data, we used two separate XIAP antibodies that are sensitive to either intact or fragmented XIAP. The application of LY 294002 enhanced the fragmentation of XIAP by TNF-α, while insulin reversed this effect (Figure 3-6B, Ab 1). Also, TNF-α treatment decreased the level of intact XIAP at 57 kDa, which was reversed by insulin and enhanced by LY 294002 (Figure 3-6B, Ab 2). In addition, insulin restored the level of intact XIAP decreased by TNF-α in a dose-dependent manner (Figure 3-6C).

3.3.4. The effects of TNF-α and insulin on the co-precipitation of XIAP and procaspase-9

Since the agents affecting the cleavage of procaspase-9 changed the level of intact XIAP, we examined the interaction of XIAP with caspase-9. Contrary to previous

literature showing that XIAP binds only to the cleaved form of caspase-9 (65, 69), we observed that XIAP was co-immunoprecipitated with procaspase-9 in control HT-29 cells (Figure 3-7A). The cleaved form of caspase-9 was not precipitated with the antibody used for the immunoprecipitation (Figure 3-7B). Based on these results, we investigated the effects of TNF-α with or without insulin on the binding of XIAP to procaspase-9. Figure 3-7C shows that TNF-α treatment decreased the level of XIAP precipitated with procaspase-9, which was reversed by insulin.

3.3.5. Protein kinase (s) responsible for the co-precipitation of procaspase-9 and XIAP

Since LY 294002 is an inhibitor of PI-3K, which is an upstream kinase of insulin signaling pathway, effects of inhibitors for downstream kinases were examined. Among three Akt inhibitors and mTOR inhibitor, rapamycin, Akt inhibitor II showed the strongest inhibition of insulin effect to decrease the TNF-α-induced cleavage of procaspase-9 (Figure 3-8A). All three Akt inhibitors are synthetic phosphatidylinositol analogues (70, 71). Akt inhibitor II and III inhibited the effect of insulin more strongly than inhibitor I, which seems to result from improved cell permeability judging from their structures. Based on this result, precipitation levels of XIAP with procaspase-9 were examined. LY 294002 and Akt inhibitor II demolished the inhibitory effect of insulin on the TNF-α-induced decrease of XIAP precipitation with procaspase-9, while rapamycin did not show any effect (Figure 3-8B). These results confirm that insulin decrease the TNF-α-induced activation of procaspase-9 by regulating XIAP via PI-3K/Akt pathway.

3.4. DISCUSSION

3.4.1. Anti-apoptotic pathway and anti-apoptotic proteins

Insulin has been reported to rescue diverse cell types from death. Two survival pathways activated by insulin are MAPKK/ERK and PI-3K/Akt pathways. Their overexpression or activation by other survival factors, such as insulin-like growth factor-1 (IGF-1), also prevents cells from undergoing apoptosis (72-74). Important questions are which anti-apoptotic proteins are affected by these kinases and which pro-apoptotic proteins are their targets. Caspases, key apoptosis mediators, accordingly, have been studied as the most likely pro-apoptotic proteins regulated by anti-apoptotic effectors, yet we still do not know complete mechanisms for survival signals to inhibit caspases. FLIP, up-regulated by survival factors (75-79), has been suggested to inhibit the activation of procaspase-8 by interfering with its binding to a death effector domain of Fas-associated death domain proteins (56-58). The literature reported conflicting data on which survival kinase regulates FLIP, probably due to different experimental conditions, including cell types. Based on our results with pharmacological inhibitors, both MAPK/ERK and PI-3K/Akt signaling pathways are involved in inhibiting the cleavage of procaspase-8. It might be the case that both kinases regulate FLIP in HT-29 cells at different levels or in different degrees. Alternatively, one kinase might decrease the cleavage of procaspase-8 via FLIP while the other operates via a different mechanism. We observed that insulin treatment slightly increased the protein level of FLIP (data not shown). Phosphorylation induced by survival signals has also been suggested to inhibit the activity of caspase-9. Phosphorylation of recombinant procaspase-9 at a serine residue by Akt was shown to

inhibit its activity (59), while phosphorylation of endogenous procaspase-9 at a threonine residue by MAPKK/ERK pathway was reported in a HeLa cell line (80). Based on these reports, different kinases may phosphorylate various sites of procaspase-9.

3.4.2. Involvement of PI-3K/Akt pathway in apoptosis of HT-29 cells

We observed that insulin decreased the rate of apoptosis and the cleavage of procaspase-8, -9, and -3 induced by TNF-α. Also, our experiments with kinase inhibitors demonstrated that the most likely pathway activated by insulin to inhibit the activation of procaspase-9 is the PI-3K/Akt pathway. Janes et al. (81) emphasized that, in HT-29 cells treated with TNF-α, Akt was the only kinase significantly activated over a long time period by insulin compared to other kinases (ERK, JNK1, IKK, and MK2). In addition, the activation of Akt by insulin showed a biphasic trend, which consists of early-time activation and sustained activation from 4 to 24 hour (81). By applying a PI-3K inhibitor, they confirmed that late-phase activation of Akt is important in decreasing apoptosis. Sustained activity of Akt in this report agrees with our observation that insulin decreased the TNF-α-induced apoptosis by reducing the cleavage of procaspase-9, a late apoptotic event, via the PI-3K/Akt pathway.

3.4.3. XIAP is responsible in anti-apoptotic mechanism

These results led us to investigate mechanism (s) by which insulin reduces the cleavage of procaspase-9, one of which might be that alterations in the interaction of procaspase-9 with other proteins regulated by the PI-3K/Akt pathway may affect its cleavage. Investigating potential molecules capable of mediating the survival effect of

insulin, we found that TNF-α induced the cleavage of XIAP, which was enhanced by a PI-3K inhibitor. Considering that XIAP is an important survival molecule to inhibit caspases, particularly, bound to procaspase-9 as discussed below, its fragmentation probably accelerates the apoptotic process. Similarly, auto-ubiquitination and degradation of XIAP occurs in response to dexamethasone and etoposide (82) and a mitochondrial serine protease, Omi/HtrA2, was suggested to degrade IAPs including XIAP (83, 84). Also, a member of the inhibitor of apoptosis protein family, c-IAP1, was cleaved by caspases, producing a proapoptotic fragment (85). Activation of Akt by overexpression of FAK (51), vascular endothelial growth factor (VEGF) (86), and insulin like growth factor-1 (IGF-1) (87) increased the gene expression or protein level of XIAP. These reports correspond to our results showing that an inhibitor of PI-3K, an upstream kinase of Akt, decreased the level of intact XIAP and induced its fragmentation. Akt was also shown to undergo cleavage during the apoptotic process (43, 88).

3.4.4. Comparison of our results and previous literature

We observed that XIAP was co-immunoprecipitated with procaspase-9. Our observation of XIAP binding to the proform of caspase-9 can explain why insulin decreased both the level of cleaved caspase-9 as well as the cleavage of its downstream substrates. If insulin affected only the cleaved form of caspase-9 via interaction with XIAP, it should alter only the level of downstream substrates of caspase-9. The possibility of procaspase-9 binding to XIAP was mentioned in earlier literature in the field (63), but later reports emphasized that XIAP binds only to the cleaved form of caspase-9 in either a cell-free system with mutated procaspase-9 or in an XIAP

overexpressed cell line (65, 69). On the other hand, we investigated the wild type endogenous procaspase-9 and XIAP from HT-29 cells. These differences in experimental protocols could lead to different results. The antibody that we used does not immunoprecipitate the cleaved form of caspase-9 (Figure 3-7B), but we cannot exclude a possibility that XIAP binds to both the proform and the cleaved form of caspase-9. Moreover, Western blotting of XIAP following immunoprecipitation of procaspase-9 demonstrated that some amount of XIAP was not precipitated with procaspase-9 (Figure 3-7A), which implies that XIAP may interact with other proteins, possibly including cleaved caspase-9, for its multi-functions. Also, this antibody recognized several bands, which might be multiple forms of XIAP. Various forms of XIAP might interact with either proform or cleaved form of caspase-9.

3.5. SUMMARY

We suggest that an interaction between XIAP and procaspase-9 is one of the regulatory systems by which insulin decreases the TNF-α-induced cleavage of procaspase-9 and the subsequent apoptosis. Our results, along with those of Janes et al. (81), strongly support an anti-apoptotic mechanism in which insulin acts through the PI-3K/Akt pathway. Further, a phosphorylation event (s) on an Akt substrate (s) may prevent release of XIAP from procaspase-9. Procaspase-9 is a potential substrate for Akt (59) and phosphorylation of XIAP by Akt was also reported recently (89). Therefore, it is feasible that phosphorylation of either or both procaspase-9 and XIAP by Akt plays a role in their interaction (and possibly other inhibitory proteins) and thus inhibits subsequent XIAP cleavage. Phosphoproteomics approach was attempted to identify phosphorylation of endogenous procaspase-9 and XIAP, but did not produce successful result due to their low abundance and significant loss during the procedure. The results shown in this chapter, in addition to the Chapter 2, demonstrating regulation of procaspase-9 via S-nitrosation in HT-29 cells (90), suggest that there are multiple inhibitory factors regulating a single component in the caspase cascade leading to apoptosis in cultured cancer cells.

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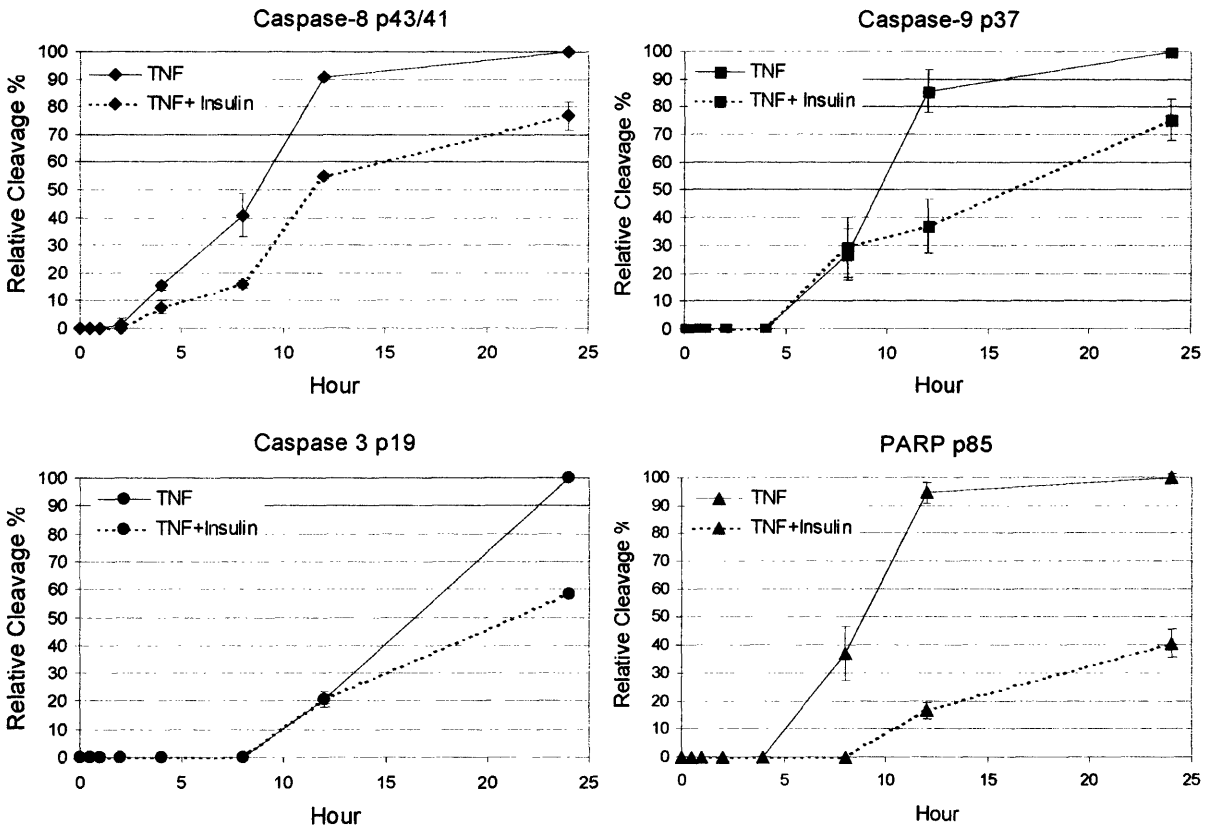

Figure 3-1. Time course of cleaved caspases and PARP induced by TNF-α with or without insulin. The cleaved forms of caspase-8, -9, -3, and PARP were detected by Western blotting from the cells treated with 50 ng/ml of TNF-α with or without 200 nM of insulin for the indicated hours following the incubation with 200 U/ml of IFN-γ for 24 hours. p41/43 of caspase-8, p37 of caspase-9, p17 of caspse-3, and p85 of PARP are their cleaved forms. Density of each band was measured by using Scion Image software and plotted with the TNF-α-induced cleavage of each protein at 24 hour point as 100%.

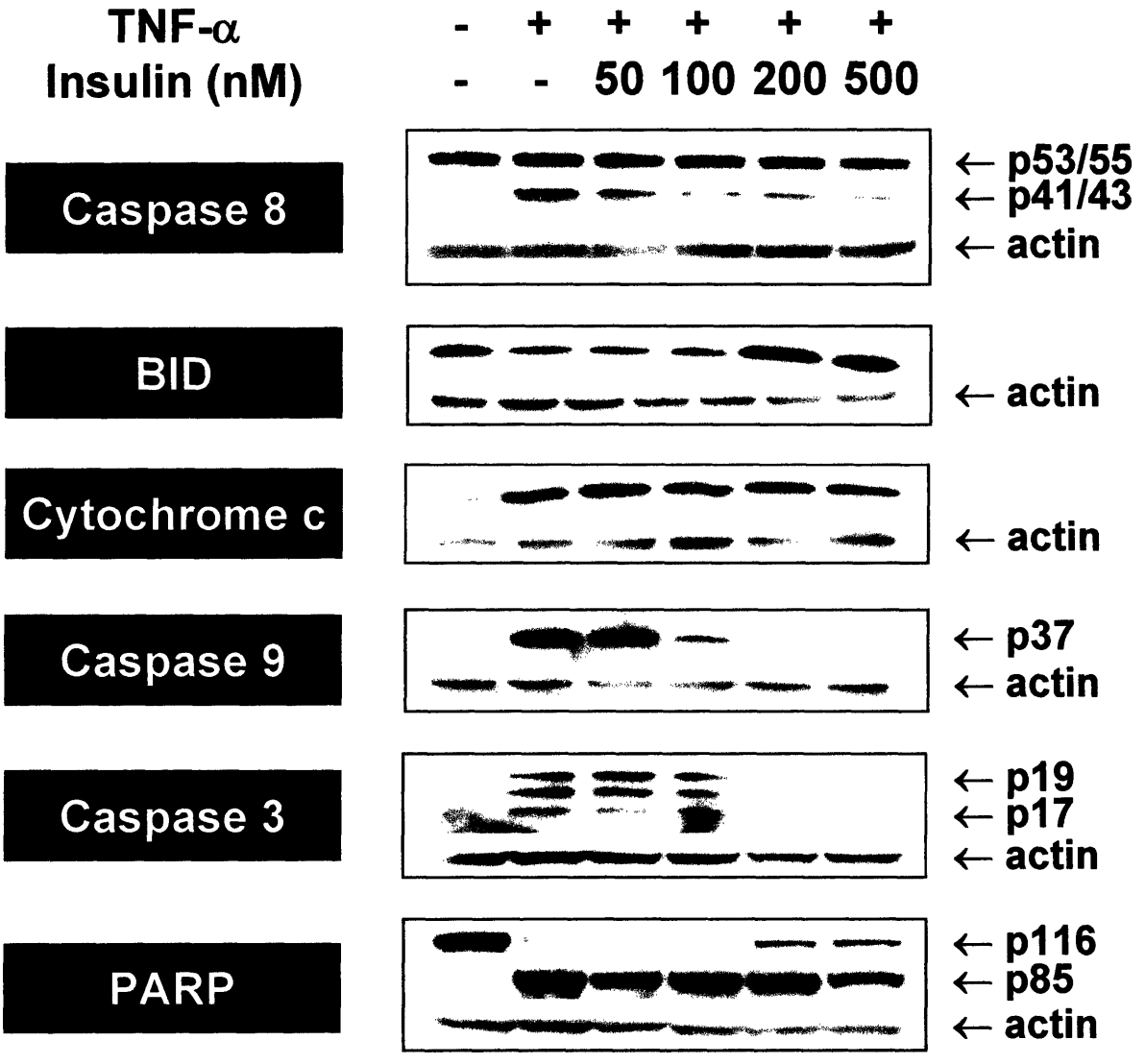

Figure 3-2. The effects of different doses of insulin on the TNF-α-induced cleavage of apoptosis-related proteins. The proform and/or cleaved form of each protein were detected by Western blotting from the cells treated with 50 ng/ml of TNF-α with or without the indicated concentrations of insulin for 8 hours following the incubation with 200 U/ml of IFN-γ for 24 hours. p53/55 of caspase-8 and p116 of PARP are their intact forms. p41/43 of caspase-8, p37 of caspase-9, p17/19 of caspse-3, and p85 of PARP represent their partial/complete cleaved forms. Bands of BID and cytosolic cytochrome *c* represent their intact forms. Actin was blotted as a loading control. The figures represent three similar experimental results.

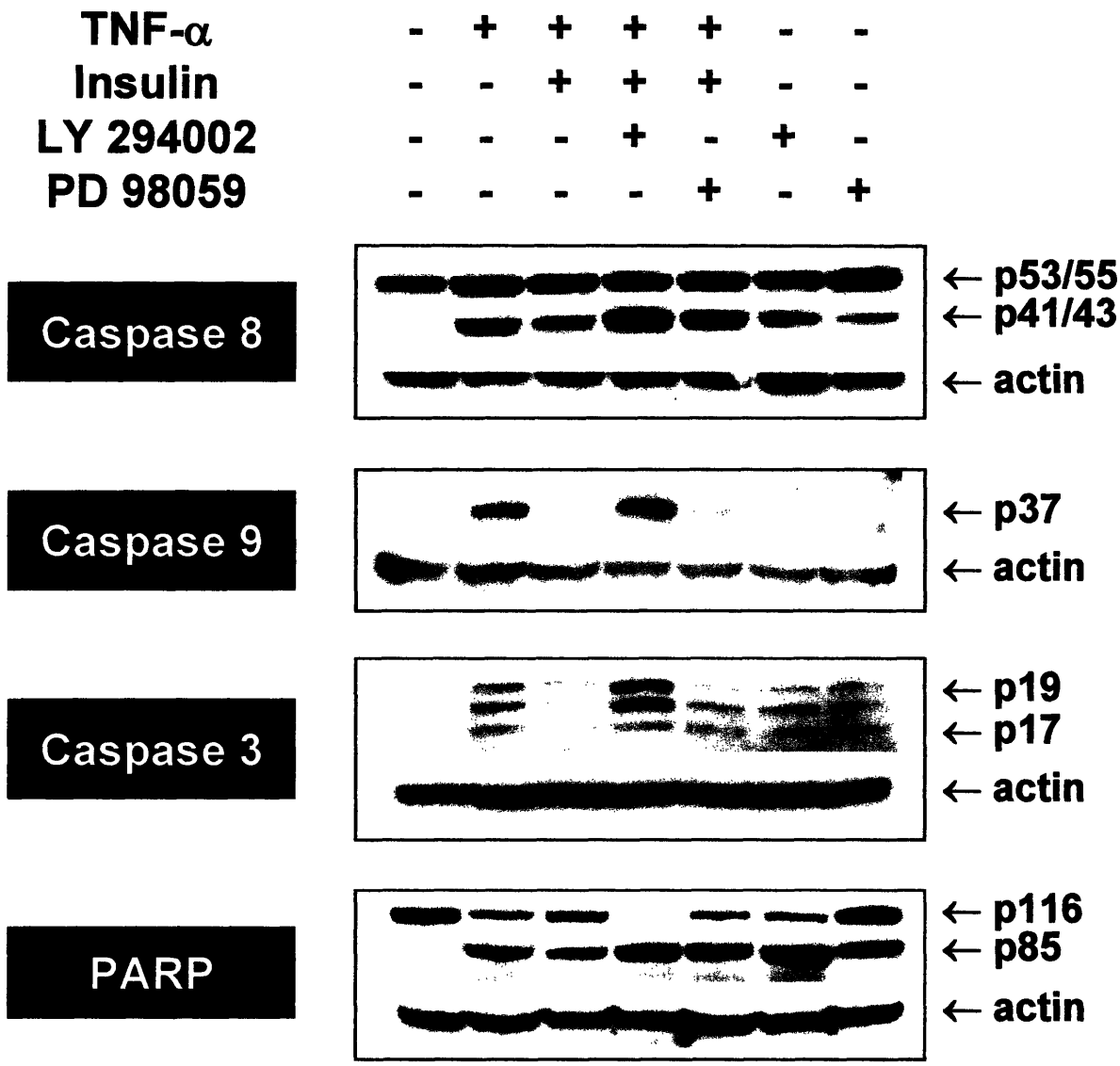

Figure 3-3. The effects of protein kinase inhibitors on apoptosis-related proteins regulated by TNF-α and insulin. The proform and/or cleaved form of each protein were detected by Western blotting from the cells treated with 50 ng/ml of TNF-α with or without 500 nM of insulin for 8 hours, in some cases, following the pretreatment with either 20 μM of LY 294002 or PD 98059 for one hour. p53/55 of caspase-8 and p116 of PARP are their intact forms. p41/43 of caspase-8, p37 of caspase-9, p17/19 of caspse-3, and p85 of PARP represent their partial/complete cleaved forms. Actin was blotted as a loading control. The figures represent three similar experimental results.

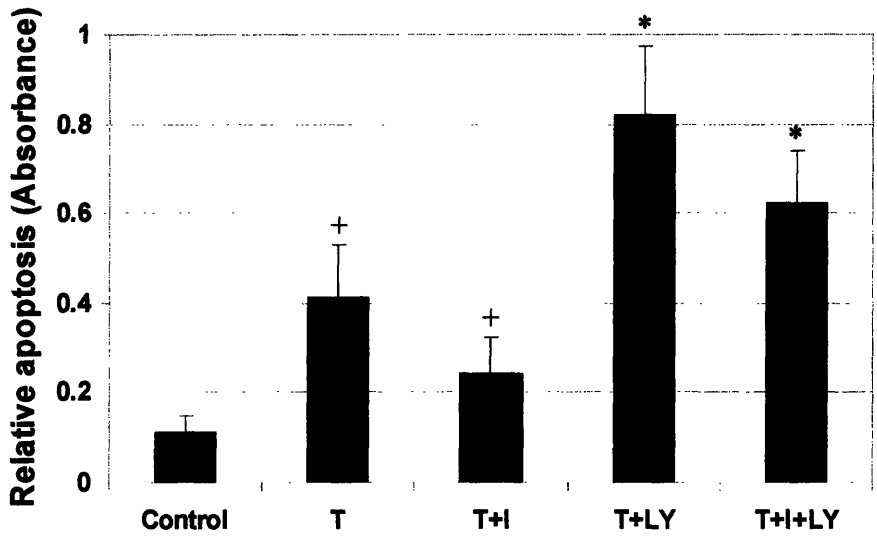

Figure 3-4. The effects of insulin and a PI-3K inhibitor on the TNF-α-induced apoptosis. Apoptotic levels were measured by ELISA assay for cytosolic histone-associated DNA fragment. Cells were treated with 50 ng/ml of TNF-α (T) with or without 500 nM of insulin (I) for 24 hours, in some cases, with the pretreatment with 20 μM of LY 294002 (LY) for one hour. * represents that the value is statistically significant at $p < 0.01$ level. + represents that the value is statistically significant at $p < 0.05$ level. Paired Student t-test with n=8.

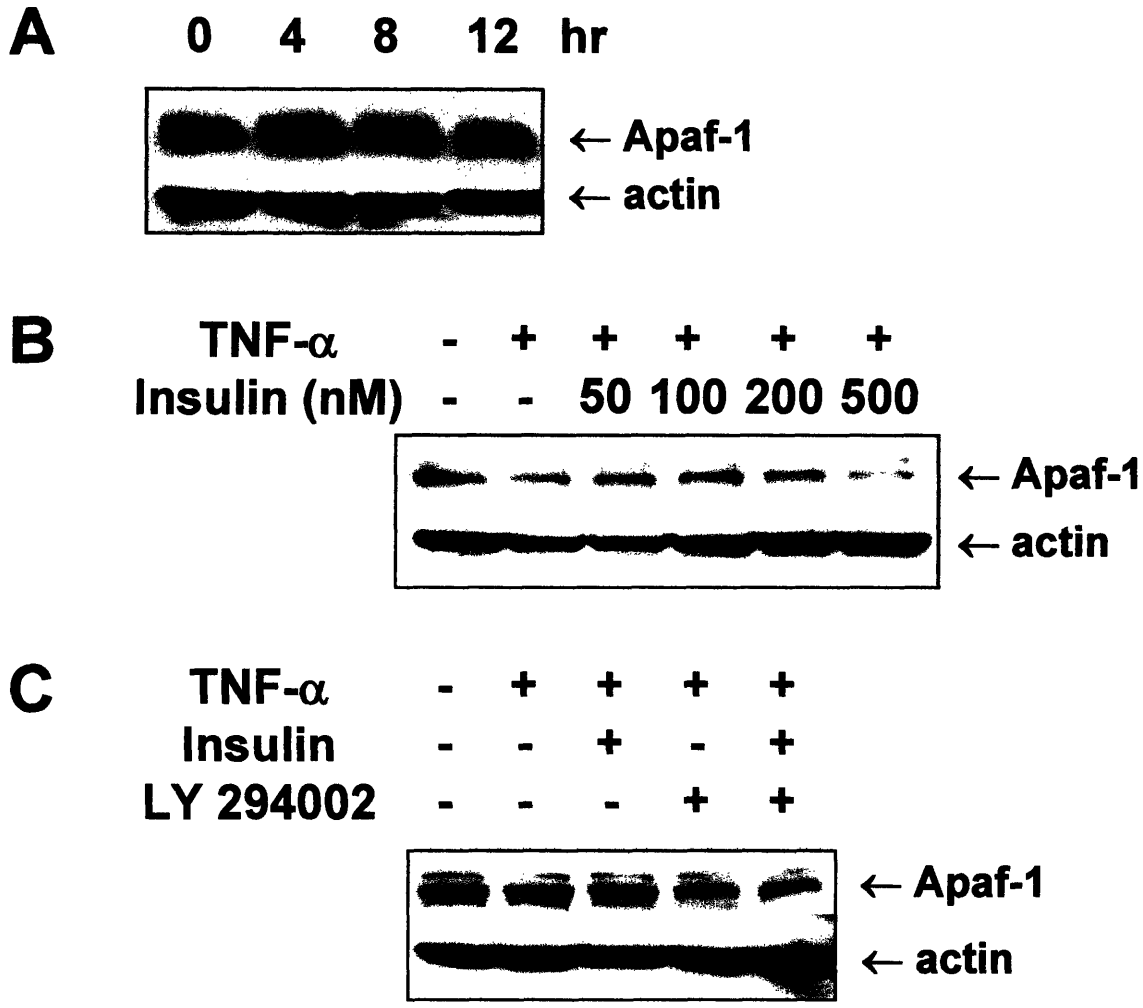

Figure 3-5. The level of Apaf-1, an activator of caspase-9, was constant with chemical treatment. *Panel A.* The protein level of Apaf-1 was detected after the incubation with 50 ng/ml of TNF-α for the indicated hours. *Panel B.* Apaf-1 protein was detected by Western blotting from the cells treated with 50 ng/ml of TNF-α with or without the indicated concentrations of insulin for 8 hours following the incubation with 200 U/ml of IFN-γ. *Panel C.* Apaf-1 protein was detected by Western blotting from the cells incubated with 50 ng/ml of TNF-α with or without 500 nM of insulin for 8 hours, in some cases, following the pretreatment with 20 μM LY 294002 for one hour. Actin was blotted as a loading control. The figures represent three similar experimental results.

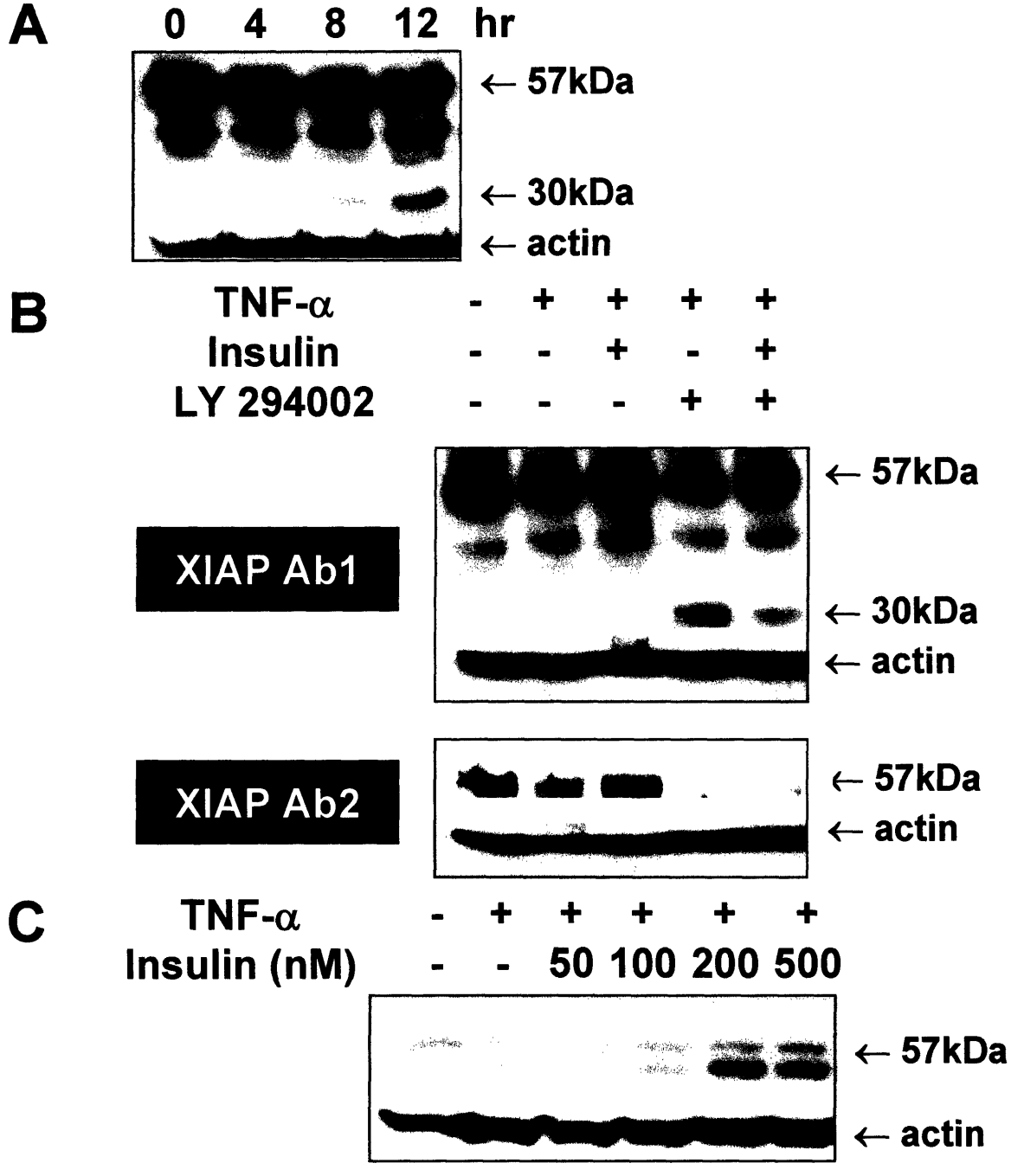

Figure 3-6. The effects of the chemicals affecting the cleavage of caspase-9 on XIAP. *Panel A.* The protein level of XIAP was detected by Western blotting from the cells incubated with 50 ng/ml of TNF-α for the indicated hours. *Panel B.* Intact and the cleaved form of XIAP were detected by two different antibodies after 8 hours of the application of 50 ng/ml of TNF-α with or without 500 nM of insulin, in some cases, following the pretreatment with 20 μM LY 294002 for one hour. Antibody 1 is anti-mouse XIAP antibody (BD Bioscience) and antibody 2 is anti-rabbit XIAP antibody (Cell Signaling Technology). *Panel C.* Intact form of XIAP was detected by using Western blotting from the cells incubated with 50 ng/ml of TNF-α with or without the indicated concentrations of insulin for 8 hours following the treatment with 200 U/ml of IFN-γ. Actin was blotted as a loading control. The figures represent three similar experimental results.

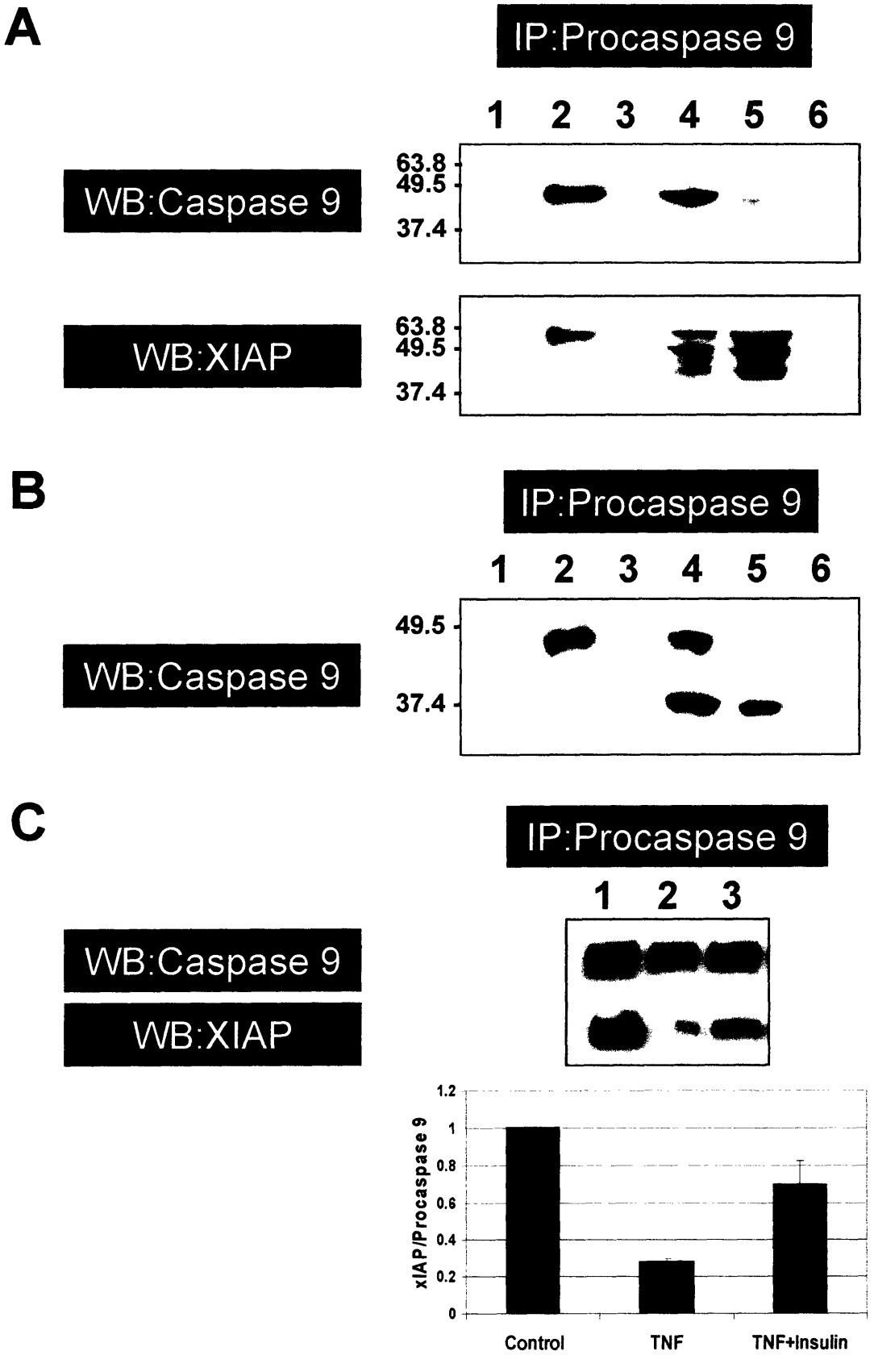
A
IP:Procaspase 9
1 2 3 4 5 6
63.8
49.5
37.4
WB:Caspase 9
63.8
49.5
37.4
WB:XIAP
B
IP:Procaspase 9
1 2 3 4 5 6
49.5
37.4
WB:Caspase 9
C
IP:Procaspase 9
1 2 3
WB:Caspase 9
WB:XIAP
1.2
1
0.8
0.6
0.4
0.2
0
xIAP/Procaspase 9
Control
TNF
TNF+Insulin

Figure 3-7. The effects of TNF-α and insulin on the co-precipitation of procaspase-9 and XIAP. *Panel A.* Procaspase-9 and XIAP were detected from the control cell lysates by Western blotting after immunoprecipitation with procaspase-9 antibody. lane 1: cell lysates precipitated with control beads, lane 2: cell lysates precipitated with antibody-immobilized beads, lane 3: no cell lysates precipitated with antibody-immobilized beads, lane 4: supernatant of lane 1, lane 5: supernatant of lane 2, lane 6: supernatant of lane 3. *Panel B.* Caspase-9 was detected from the control cell lysates with an antibody that recognizes both proform and cleaved form after immunoprecipitation with procaspase-9 antibody. lane 1: cell lysates precipitated with control beads, lane 2: cell lysates precipitated with antibody-immobilized beads, lane 3: no cell lysates precipitated with antibody-immobilized beads, lane 4: supernatant of lane 1, lane 5: supernatant of lane 2, lane 6: supernatant of lane 3. *Panel C.* Caspase-9 and XIAP were detected by Western blotting after immunoprecipitation of procaspase-9 from the HT-29 cells incubated with 50 ng/ml of TNF-α with or without 500 nM of insulin for 12 hours following 200 U/ml of IFN-γ. The ratio of XIAP and procaspase-9 was calculated from the density of bands measured by using Scion Image software. The figures represent three similar experimental results.

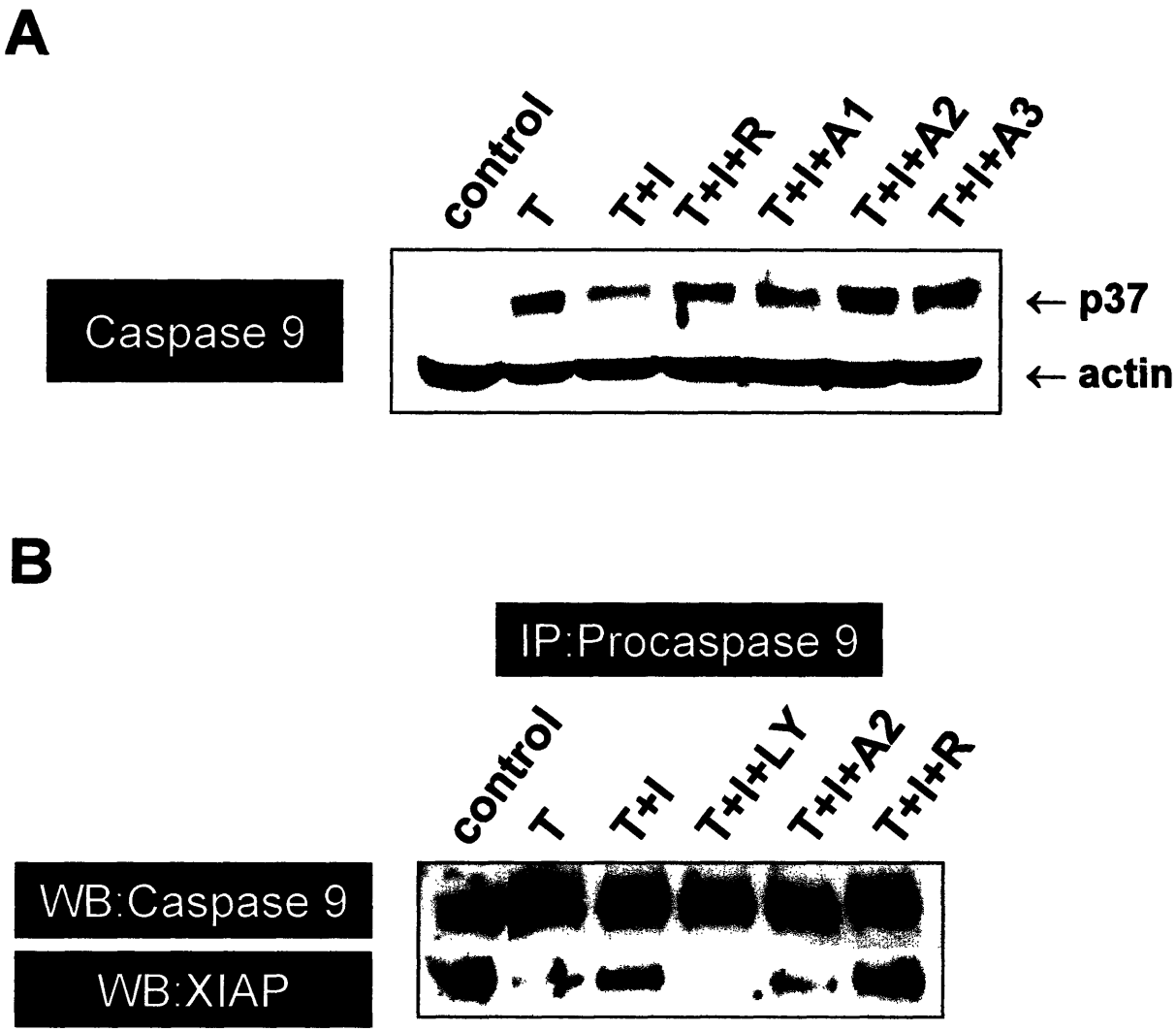

Figure 3-8. The effects of kinase inhibitors on the precipitation of procaspase-9 and XIAP. *Panel A.* The protein level of cleaved caspase-9 was detected by Western blotting from the cells incubated with 50 ng/ml of TNF-α for 8 hours with or without insulin following the pretreatment of kinase inhibitors for 1 hour. R: 200 nM rapamycin; A1: 20 μM of Akt inhibitor I; A2: 20 μM of Akt inhibitor II; A3: 20 μM of Akt inhibitor III. *Panel B.* Caspase-9 and XIAP were detected by Western blotting after immunoprecipitation of procaspase-9 from the HT-29 cells incubated with 50 ng/ml of TNF-α with or without 500 nM of insulin for 12 hours following the pretreatment of kinase inhibitors for 1 hour. LY: 20 μM of LY 294002 R: 200 nM of rapamycin; A2: 20 μM of Akt inhibitor II.

Chapter 4. CHARACTERIZATION OF PHOSPHOPROTEINS REGULATED BY AN APOPTOTIC STIMULUS, TUMOR NECROSIS FACTOR-α, IN HT-29 CELL LINE

4.0. ABSTRACT

Phosphorylation events in signaling cascades triggered by a variety of cellular stimuli modulate functions of proteins, leading to diverse cellular outcomes including cell division, growth, death, and differentiation. Abnormal regulation of phosphorylation due to mutation or overexpression, therefore, results in disease states. As a preliminary study for further investigation of phosphoproteins regulated in responses to diverse cellular signals, whole cell phosphopeptides were identified in a human colon adenocarcinoma cell line, HT-29, treated with insulin, by employing a phosphoproteomics technique with liquid chromatography (LC)-mass spectrometry (MS)/MS. Whole HT-29 cell protein extracts were digested with trypsin followed by conversion of carboxylate groups to methyl esters. Derivatized phosphopeptides were enriched using Immobilized Metal Ion Affinity Chromatography (IMAC). Phosphopeptides were, in turn, separated by high performance liquid chromatography (HPLC) and analyzed by electrospray ionization-quadrupole-time-of-flight (Q-STAR®) mass spectrometry. Database search by MASCOT algorithm followed by manual confirmation of peptide sequences as well as phosphorylation sites enabled us to characterize 176 confirmed and 22 potential phosphorylation sites in 114 phosphopeptides. Additionally, we identified 28 phosphopeptides containing 64 potential phosphosites, but could not locate phosphorylation sites. In addition to a search of published literature, Scansite was used to search possible kinases for each phosphopeptide. We also investigated phosphopeptides regulated by an extracellular apoptotic signal, tumor necrosis factor-α. At two time points of post-stimulation, we could detect alteration of some apoptosis-related proteins.

Phosphorylation states of these proteins may regulate the apoptotic process. Proteome-wide IMAC-LC/MS/MS approach enabled us to identify some low-abundance proteins and to detect phosphoproteins possibly regulated during apoptosis. However, in order to obtain quantitative information on the higher number of low-abundance proteins, additional sample preparation will be necessary to simplify and enrich sample mixture.

4.1. INTRODUCTION

Phosphorylation is a crucial modification to regulate functions of proteins involved in signaling pathways. Aberrant regulation of phosphorylation, accordingly, leads to disease states (1, 2). Thus, abnormal activity of protein kinases and phosphatases resulting from overexpression or mutations has been reported in carcinogenesis (2). Mapping components and their regulations in signaling pathways, such as kinases, phosphatases, and their substrates, particularly, oncogenes or tumor suppressor genes, therefore, has been extensively studied. Traditionally, ^{32}P-based protein blotting and phosphopeptide mapping combined with Edman sequencing visualize phosphoproteins and identify modification sites. These procedures require intensive labor, radioactive materials, and often purified proteins. On the other hand, proteomics technology has focused on developing methods to detect and measure a large number of phosphoproteins in shorter time, demonstrating considerable improvements in both qualitative and quantitative aspects. Nevertheless, identification of phosphoproteins or characterization of their phosphorylation sites is still challenging, although a third of all proteins in eukaryotic cells are phosphorylated at any given time (3). Separation of cell extracts with or without various treatments (4,5) using one or two dimensional gel electrophoresis followed by mass spectrometry analysis is a typical proteomics procedure. This procedure was improved by enrichment of low abundance phosphoproteins with phosphospecific antibodies, particularly, phosphotyrosine antibody, followed by mass spectrometry analysis (6). Still, proteins phosphorylated at tyrosine residues comprise only 0.05% of all phosphorylated proteins (7). In addition, phosphoserine/threonine

proteins are still difficult to analyze with this approach due to lack of efficient antibodies despite recent improvement (8, 9). Moreover, isolation and identification of phosphopeptides in peptide mixtures revealed disadvantages in locating specific phosphorylation sites because of ionic suppression in the presence of non-phosphorylated peptides. Precursor ion scanning mode facilitated detection of phospho-tyrosine residues, but not phospho-serine/threonine residues due to their lability (10-14). These limitations led different research groups to develop methods to enrich phosphopeptides instead of phosphoproteins. Oda et al. isolated and identified phosphopeptides by labeling phosphate moieties with biotin following β-elimination (15). Zhou et al. also captured phosphopeptides via covalent bonds to glass beads after adding sulfhydryl to phosphate moieties (16). These methods enhanced specificity, but require multi-step sample preparations and detected only high-abundance proteins possibly due to sample loss from harsh experimental conditions. Other challenges in phosphoproteomics using mass spectrometry are lability of phosphate groups in collision-induced-dissociation mode and difficulty in obtaining full sequence coverage. Recent techniques designed by Knight et al. introduced chemical transformation of phospho-serine/threonine into lysine analogs using β-elimination followed by a reaction with aminoethylcysteine, resulting in phosphospecific cleavage, unique y_1 ions, and consequently improved MS/MS data interpretation (17). This technology, however, cannot distinguish phosphorylation from O-glycosylation unless combined with phosphatase or glycosidase pretreatment. Also, this method is still limited to only phospho-serine/threonine residues and its application to biological samples was not performed yet. Ficarro et al. combined nano liquid chromatography (LC)/mass spectrometry (MS) technique and Immobilized Metal Ion

Affinity Chromatography (IMAC), which was developed and applied by various groups (18-25), to characterize enriched phosphopeptides from yeast (26). Conversion of carboxylate groups into methyl esters reduced non-specific binding to IMAC, thereby resulting in a high coverage of phosphopeptides including low-abundance phosphoproteins from Saccharomyces Cerevisiae. This method involves only a single step and detects all three phosphorylation forms.

Since it was shown that quantitative information on proteins cannot be predicted from mRNA expression data (27), the proteomics field has focused on the development of quantitative methods to measure both expression and function of proteins. Traditionally, isotope-coded alkylating agents have been used to obtain quantitative information. Isotope-Coded Affinity Tag (ICAT) developed by Gygi et al. (28) was a breakthrough report combining biotin labeling to isolate cysteine-containing peptides and thereby to simplify a mixture introduced to LC/MS system and traditional isotope effects for quantitation. This report, however, presented mostly high abundance proteins. Solid-phase photocleavable isotope tagging, an improved method based on ICAT, identified a larger number of proteins than the previous report (29). Mass-coded abundance tagging (MCAT) introduced an idea to modify lysine residues with O-methylisourea into homoarginine to compare with unmodified peptides (30), and isotope-based metabolic labeling in cell culture systems has also been reported. Conrads et al. combined cysteine affinity tags and metabolic labeling for more complementary information (31).

Alterations in phosphorylation states of proteins are often used as indicators of cell fate-growth, division, apoptosis, or differentiation. Accordingly, quantitative methodologies to compare different states of cells for identification of biomarkers or

different chemical treatments for testing drug efficacy have been developed. Steinberg et al. has reported a fluorescent dye named Pro-Q Diamond combined with two dimensional gel electrophoresis to quantitate phospho-proteins (32). This dye can provide quantitative information of phosphorylation in proteins, but not of phosphorylation sites, which means lack of information on regulation at multiple phosphorylation sites in responses to cellular stimuli. Difficulties in identifying phosphopeptides from peptide mixtures, even from a protein, led researchers to develop diverse methods to enrich phosphopeptides. A method to enrich and quantitate phosphopeptides at the same time has been developed from traditional isotope-coded affinity tag after β-elimination at phosphorylation sites (33-35). This approach reported only a small number of phosphoproteins from the MCF-7 cell line without comparison of different cell states. Isotope-labeled amino acids, e.g., ^{13}C and ^{15}N, in culture medium also have been used for quantitation (36, 37). This literature reported quantitative information on only a specific protein, not global level.

No literature has provided phosphoproteomics information on a human colon adenocarcinoma cell line, HT-29 cells. Applying IMAC followed by nano LC/MS/MS, we now demonstrate a profile of phosphopeptides and their modification sites from HT-29 treated with insulin. We also detected phosphopeptides potentially regulated during the early apoptotic process induced by tumor necrosis factor-α. Additional sample preparation is, however, necessary for relative quantitation of a larger number of low-abundance proteins.

4.2. EXPERIMENTAL PROCEDURES

4.2.1. Cell culture and chemical treatments

Human colon epithelial adenocarcinoma cell line, HT-29 (provided from the Peter Sorger laboratory in the MIT Biology Department), was maintained in McCoy's 5A medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and L-glutamine (Life Technologies) at 37°C under 5% CO_2. Cells were seeded onto culture plates at a density of $5x10^4/cm^2$ and grown for 24 hours. Then, 200 U/ml of interferon-γ (IFN-γ) (Roche Applied Science, Indianapolis, IN) was applied for 24 hours, followed by treatment with 500 nM of insulin and 1 μM of okadaic acid (Sigma, St. Louis, MO) for 30 minutes. For apoptotic cell analysis, cells were treated with 50 ng/ml of tumor necrosis factor-α for the indicated hours following the application of interferon-γ to sensitize the cell line to death signal.

4.2.2. Protein extraction, digestion, and esterification

Whole cell proteins were extracted from $5x10^6$ of HT-29 cell lysates using Trizol® (Life Technologies) according to the manufacturer's instruction. The protein pellet was resolubilized in 1% of SDS and diluted to 0.2% of SDS by adding 100 mM of ammonium acetate (pH. 8.9). Then, proteins were digested with 20 μg of trypsin (Promega, Madison, WI) overnight at 37 °C. Digested peptides were lyophilized in Speed Vac and then derivatized with 1 ml of anhydrous methanol and 40 μl of thionyl chloride for two hours followed by lyophilization.

4.2.3. Enrichment of phosphopeptides by IMAC

IMAC was prepared as described (26). Briefly, 15 cm long (750 μm o.d. x 530 μm i.d.) microcapillary fused-silica column (Polymicro Technologies, Phoenix, AZ) was packed with POROS 20MC (Applied Biosystems, Framingham, MA) and washed with 100 mM of [ethylenedinitrilo]-tetraacetic acid (EDTA). Then, the column was loaded with 100 mM of $FeCl_3$ for 10 minutes at 100 p.s.i. Next, derivatized peptides from ~$1x10^6$ cells were loaded into the IMAC column followed by washing with an organic buffer (25 % acetonitrile, 100 mM NaCl, and 1 % acetic acid) and 0.1 % acetic acid. Phosphopeptides were eluted with 250 mM of NaH_2PO_4 into an 8 cm long (360 μm o.d. x 100 μm i.d.) microcapillary fused silica precolumn packed with 10 μm C18 particles (YMC, Wilmington, NC).

4.2.4. Mass spectrometry analysis

The precolumn was connected to a 10 cm long (360 μm o.d. x 50 μm i.d.) microcapillary fused silica analytical column with an integrated electrospray ionization tip (~1μm) and packed with 5 μm C18 particles (YMC ODS-AQ, Waters, MA). Phosphopeptides were analyzed by HPLC-electrospray ionization-quadruple-time-of-flight system (Applied Biosystems) with a gradient of 0-60 % B in 200 minutes, 60-100 % B in 10 minutes, 100-100 % in 2 minutes, and 100-0 % in 2 minutes with a flow rate less than 50 nl/min. The instrument cycled through acquisition of a full-scan mass spectrum (m/z 400-1500) for a second followed by 3 MS/MS scans (charge state 2 - 5+, m/z 100-2000, accumulation time 1.5 sec, exclusion time of former target ions 60 seconds, resolution set as low, collision energy = slope*(m/z) + intercept with maximum

80 V; charge state/slope/intercept; 2+/0.0325/15; 3+/0.0300/9; +4/0.0300/7) sequentially on the three most abundant ions present in the initial MS scan.

4.2.5. Database analysis

All MS/MS spectra were converted for subsequent search against human protein database by using MASCOT algorithm. Search parameters contained a variable modification of +80 Da on serine, threonine, and tyrosine, β-elimination of phospho amino acids, and a fixed modification of +14 Da on the C-terminal of peptides and the side chains of aspartic acid and glutamic acid. Then, phosphorylation sites and sequence of identified peptides in MASCOT search results were validated by confirmation from raw MS/MS data.

4.3. RESULTS

4.3.1 Identification of phosphopeptides from HT-29 cells treated with insulin

Table 4-1 shows a list of confirmed phosphopeptides from HT-29 cells treated with insulin. Most phosphopeptides identified with high score in this experiment are from high-abundance proteins, which are involved in structural maintenance, transcription, mRNA processing, and translation. However, proteins involved in signaling pathways such as p53, CDC 2 isoform 1, PDGFA associated protein-1, Protein kinase D2, PKA alpha 1 catalytic subunit, AP2 associated kinase-1 were also identified. We observed a number of singly phosphorylated peptides, which suggests that the column was not overloaded. We excluded any hypothetical protein, of which function was not published. There is a list of phosphopeptides with unconfirmed phosphorylation sites (Table 4-2). Some of the peptides were detected only from insulin-treated cells (Table 4-1). In summary, a database search with MS/MS data by MASCOT algorithm followed by manual confirmation of peptide sequences, as well as phosphorylation sites, showed 176 confirmed and 22 potential phosphorylation sites in 114 phosphopeptides from HT-29 cells treated with insulin. Additionally, 28 phosphopeptides were detected although we could not locate potential 64 phosphosites due to insufficient MS/MS data.

4.3.2. Detection of phosphopeptides potentially regulated during early apoptosis

It is known that both phosphorylation and dephosphorylation of proteins occur during apoptosis and are also involved in regulating apoptosis. A question is whether there is a general trend of (de) phosphorylation to explain mechanism of apoptosis.

Given that apoptosis is a multi-step and complex process, it is likely that regulation of phosphorylation should be investigated both at the level of individual protein and global protein networks instead of drawing one conclusion.

Table 4-3, 4-4, and 4-5 present phosphopeptides detected at 0, 4, and 8 hour after incubation with tumor necrosis factor-α (TNF-α). Next, Table 4-6 shows a list of phosphopeptides detected at 0 and 4 hour while Table 4-7 shows phosphopeptides detected at 4 hour and 8 hour. Results suggest that regulations of phosphorylation vary among phosphoproteins, therefore focus should be to examine functional outcome of each protein when it is either phosphorylated or dephosphorylated. Information on unknown phosphorylation sites of apoptosis-related proteins from our data support that altered phosphorylation states of both anti- and pro-apoptotic proteins regulate the apoptotic process. BCA protein assay shows little changes in total protein concentration (Figure 4-1). On the other hand, both cleavage of some caspases (Figure 3-1) and apoptosis start to increase at 8 hour time point (Figure 4-1). These results suggest that protein degradation does not interfere with interpretations of altered phosphorylation during apoptosis.

4.4. DISCUSSION

4.4.1. Advantages and disadvantages of global level phosphoproteomics

Development of IMAC in the format of microcapillary column chromatography combined with LC/MS/MS facilitated a large amount of data in phosphoproteomics field. Datasets start to be established in diverse systems and efforts to develop and apply quantitative methods to physiological systems (e.g., cell lines, primary cells, and tissues) have been made. The lists of phosphopeptides from HT-29 cells in this thesis show a large number of phosphopeptides from the smaller number of cells than previously published reports. Also, the lists include signaling proteins, such as p53, CDC 2 isoform 1, PDGFA associated protein-1, Protein kinase D2, PKA alpha 1 catalytic subunit, AP2 associated kinase-1. There are, however, still limitations in the present phosphoproteomics techniques in identifying low-abundance, signaling phosphopeptides from a global level analysis. We attempted to obtain quantitative information by derivatizing carboxyl groups including aspartate and glutamate with D_0 and D_3 isotope-labeled agents. This approach with whole cell proteins showed some drawbacks in studying low-abundance proteins. Although extracted ion chromatography enabled us to quantitate abundant peptides with distinct peaks, it is hard to compare peaks of low-abundance peptides in a complex mixture such as whole cell proteins, demonstrating issue of dynamic range in the global level approach. Based on the results, in order to identify a specific pathway or low-abundance signaling proteins, it is necessary to simplify samples and, thereby, enrich target proteins to obtain complementary information in addition to global level data.

4.4.2. General trend of phosphopeptides potentially regulated during apoptosis

The apoptosis mechanism is very complex and is regulated by both phosphorylation and dephosphorylation, which may cause or block apoptosis in the appropriate context. Our results suggest that regulation of phosphorylation events vary among proteins at each time point of apoptosis, emphasizing interpretation at individual protein level. Our results with unknown phosphorylation sites from apoptosis-related proteins provide complementary information to known regulation of anti- and pro-apoptotic proteins. Functions of those proteins whose detection was altered by tumor necrosis factor-α are discussed next.

4.4.3. Functions of phosphoproteins (un) detected during apoptosis

A number of phosphopeptides were detected at different time points. Since relative quantification in this approach showed some challenges, only on or off detection was considered and listed in this thesis. Among the phosphopeptides in tables, proteins related to apoptosis are discussed here for their functions. TGF-β1-induced anti-apoptotic factor-1 (TIAF-1), caspase-8 associated protein-2 (FLASH homolog RIP25), and damage-specific DNA binding protein-2 (DDB-2) were detected only at 0 hour. TGF-β1-induced anti-apoptotic factor inhibits apoptosis induced by both TNF-α and overexpression of TRADD, FADD and RIP (38). Also, it mediates inhibition of IκB-α expression by TGF-β and inhibits TNF-α-mediated IκB-α degradation (38). The role of TIAF-1, however, seems to vary among cell types (39). Caspase-8 associated protein-2 (FLASH homolog RIP25) is a homologue to FLASH, a mouse apoptotic protein, which interacts with the death-effector domain (DED) of caspase-8 and may be a component of

the death-inducing signaling complex (DISC) (40). Also, caspase-8 associated protein-2 coordinates NF-κB activity induced by TNF-α (41). Damage-specific DNA binding protein-2 (DDB-2) is involved in repair of UV-induced apoptosis and confers cells resistance to UV (42). DDB-2 and p53 regulates mutually (43). Although our apoptotic agent was a death receptor ligand, DDB-2 could be related to chromosomal changes during apoptosis.

Cathepsin C (dipeptidyl-peptidase I) is required for the proteolytic activation of progranzymes B, whose activation leads to partial processing of procapase-3 (44). 3-Phosphoinositide dependent protein kinase-1 (PDK-1) plays an important role in regulating the Akt survival pathway by phosphorylating Akt at Thr-308 (45). PDK-1 is auto-phosphorylated at Ser-241 (46), which was identified in our experiment and may be negatively regulated by binding to 14-3-3 (47). Phosphorylation of Akt, a substrate of PDK-1, by TNF-α was shown (48), but, in general, apoptotic signals block phosphorylation of Akt and degrade Akt (49).

Death-associated protein (DAP) is a basic, proline-rich, 15-kD protein, which was identified as a positive mediator of programmed cell death induced by interferon-γ (50). Interferon-γ was used as a sensitizing agent to apoptosis in our system. It is feasible that expression of DAP is induced by one cytokine and then it may be phosphorylated by another cytokine as apoptosis progresses. In our result, DAP was detected at 8 hour. A proapoptotic factor, interferon-inducible double stranded RNA dependent protein kinase, interacts with apoptosis signal-regulating kinase-1 (ASK-1), involved in apoptotic signaling pathway (51). This protein was detected at both 4 and 8 hour.

A lot of phosphopeptides were not detected at 8 hour compared to earlier time points. Apoptosis inhibitor 5, also named as FGF-2-interacting-factor (FIF) (52) or 55-kDa AAC-11 protein (53), is an example. Also, baculoviral IAP repeat-containing 6 is a human IAP-family gene, apollon, expressed in human brain cancer cells (54). This protein may exert cell protective mechanism from apoptosis. IAP-associated factor VIAF-1, also called phosducin-like 3 (55), was not detected. MAPK 14 (p38 MAP kinase) may be involved in the early onset of apoptosis (56-60). We could identify known phosphorylation sites of p38 MAP kinase, both Thr-180 and Tyr-182, at 0 and 4 hour. We could not quantitate p38 phosphorylation, but function of p38 may be modulated by other proteins in control cells, considering that its phosphorylation was detected under normal conditions. Also, the fact that it was not detected at 8 hour may support its role in early onset of apoptosis. Programmed cell death-5, also called TF-1 cell apoptosis related gene-19 (TFAR-19), is suggested to play an early and universal role in apoptosis (61). SH3-domain kinase binding protein-1 (SH3KBP-1) is an 85-kD c-Cbl-interacting protein that enhances TNF-α-mediated apoptotic cell death possibly via involvement in ligand-induced downregulation of receptor tyrosine kinases, such as epidermal growth factor receptor, by Cbl (62-65). Exact function or phosphorylation sites of some apoptosis-related proteins regulated by TNF-α in our results was not previously reported. Functional outcome of altered phosphorylation is, therefore, not clear, but anti-apoptotic proteins may undergo dephosphorylation, possibly accelerating the late phase of the apoptotic process.

4.4.4. Conclusions

It is known that altered phosphorylation states of anti- and pro-apoptotic phosphoproteins regulate the apoptotic process. In support of this, our phosphoproteomics approach with IMAC-LC/MS/MS allowed us to detect alteration of phosphopeptides from apoptosis-related proteins at two time points during early apoptosis induced by tumor necrosis factor-α. In order to obtain complete information on regulation of apoptosis including identification and improved quantitation of the phosphorylation states of low-abundance proteins, it will be necessary to simplify protein samples via immunoprecipitation or fractionation.

4.5. SUMMARY

With help of IMAC combined with nano LC/MS/MS, a list of phosphopeptides from HT-29 cell line was identified. Also, the same technique enabled us to detect phosphoproteins whose alterations are potentially important in the progress of apoptosis induced by tumor necrosis factor-α. The technique provides a powerful tool to identify phosphopeptides. However, in order to quantitate a number of peptides from different cell states, further isolation of a group of interesting peptides or additional fractionation is necessary in sample preparation step.

4.6. REFERENCES

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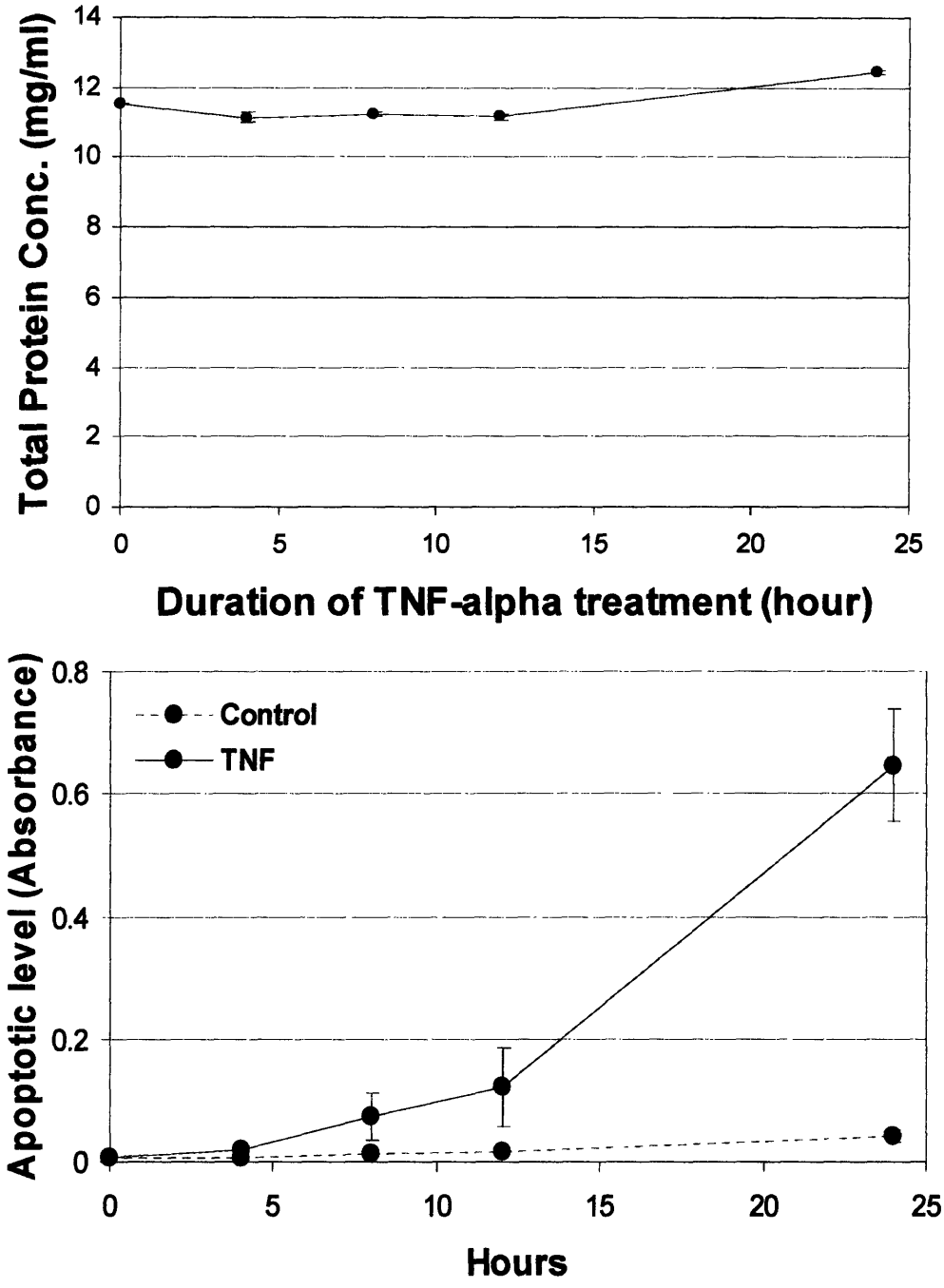

Figure 4-1. Changes of total protein concentration and apoptotic level during TNF-α treatment. *Upper chart,* Total protein concentration was measured using the bicinchoninic acid (BCA) protein assay. n=3. *Bottom chart,* Apoptosis levels were measured with cell death detection ELISA plus (Roche).

Phosphopeptides identified from HT-29 cells treated with insulin/okadaic acid

Table 4-1. Phosphopeptides with confirmed phosphorylation sites

* represents that phosphorylation site was not confirmed from raw data due to insufficient MS/MS spetrrum.
(s) represents predicted phosphorylation site and kinases from data search by Scansite (scansite.mit.edu)
Insulin-activated means that the peptide was detected with insulin-treated cells compared to control cells.

Protein	Phosphopeptides	score	Comments
Lamin A/C (gi|5031875)	KLEpSTESR	34	*Insulin-activated*
	A*SSHSpSQTQGGGSVTKKR	65	S407; During interphase
	A*SSHSSQpTQGGGSVTK	46	T409; During interphase
	GRASSHpSpSQTQGGGSVTK	47	S407
	LRLpSPpSPTSQR	18	S390;S392; cdc2-kinase; regulates mitotic lamin A disassembly
cytokeratin 8 (gi|4504919)	ISSSpSFSR	39	S36 (s) PKC delta GSRISSSSFSRVGSS
	AFSSRpSYTSGPGSR	34	S23 during mitosis; PKCε?
	AFpSpSRSYTSGPGSR	32	
type I cytoskeletal 18 (gi|37539666)	pSTFSTNYR	32	PKCε?
Ser/Arg-related nuclear matrix protein (gi|5032119)	RR*SPpSPPPTR	19	
	RYpSPpSPPPKRR	29	*Insulin-activated*
	RRpSPpSPAPPPR	18	
	HRPpSPPApTPPPK	31	*Insulin-activated*
	TRHpSPpTPQQSNR	18	
	KAApSPpSPQSVR	40	*Insulin-activated*
	KVELpSEpSEEDKGGK	23	*Insulin-activated*
	KPPAPPpSPVQ*SQSPSTNW*SPAVPVKK	30	
plectin 1 (gi|4505877)	SSpSVGpSSSSYPISPAVSR	31	p34CDC2 kinase
matrin 3 (gi|106964)	SYpSPDGKESPSDKK	29	S596 (s) PKA KDKSRKRSYSPDGKE
	SYpSPDGKEpSPSDKKSK	21	
supervillin isoform 1 (gi|4507323)	RGpSLER	15	S574 (s) PKA YAVPRRGSLERANPP
Bcl-2-associated transcription factor (gi|7661958)	STFREEpSPLRIK	36	*Insulin-activated*
	KAEGEPQEEpSPLKSK	17	*Insulin-activated*
general transcription factor IIF, polypeptide 2 (gi|4758488)	HYQGEEKpSD	20	*Insulin-activated*

general transcription factor IIF, polypeptide 1 (gi|4504197)	IHDLEDDLEMpSpSDApSDASGEEGGRVPK	39	*Insulin-activated*
elongin A (gi|4507389)	SYpSPDHR	19	S125 (s) Cdk5 kinase/ CDC2 kinase ATGSRSY**S**PDHRQKK
calcium-regulated heat-stable protein (24kD) (gi|7656995)	TFpSATVR	28	S52 (s) Akt TRRTRTF**S**ATVRASQ T54 (s) PKC αβγ RTRTFSA**T**VRASQG
	ERpSPpSPLRGNVVPpSPLPTRR	16	S30 (s) Akt TPRSRER**S**PSPLRGN *Insulin-activated*
TBP-associated factor 2 (gi|4507347)	SIRpSPpSLpSD	24	*Insulin-activated*
autoantigen La (gi|10835067)	FApSDDEHDEHDENGATGPVKR	21	S366; CK2
glucocorticoid receptor DNA binding factor 1 isoform a (gi|25121938)	KVpSIVSKPVLYRTR	20	
HIV TAT specific factor 1 (gi|166761)	ARHFpSEHPSTSK	19	
tripartite motif-containing 28 protein 1 (gi|5032179)	SRpSGEGEVSGLMR	21	
serine-arginine repressor protein (35 kDa) (gi|18034491)	QTpSSG*TKpSR	19	
Smad nuclear interacting protein (gi|21314720)	GVpSRpSPPKKK	15	S76 (s) cdk5/cdc2 RARGVSR**S**PPKKKNK *Insulin-activated*
scaffold attachment factor B (gi|21264343)	SVVpSFDKVKEPR	16	
LOMP protein (gi|4929268)	RGEpSLDNLDSPR	28	
splicing coactivator subunit SRm300 (gi|19923466)	RGpSRpSSPEPK	15	*Insulin-activated*
	RRPpSPQPpSPR	17	
	SGpSpSPEVKDKPR	33	
	THTTALAGRpSPpSPASGR	26	
	VKAIIpSPR	27	*Insulin-activated*
	pSRSPSpSpPELNNK	21	
	GKRpSLpTRpSPPAIR	18	
	SSGHpSSSELpSPDAVEK	26	
	NHpSG*SR*TPPVALNSSR	22	
	AQpSG*SDpSpSPEPKAPAPR	29	*Insulin-activated*
	SSpSASSPEMKDGLPR*TPSRR	16	S1421 (s) Akt PSRERSS**S**ASSPEMK

	pSGTPPRQGSITSPQANEQSVpTPQRR SATRPpSPpSPERSSTGPEPPAPTPLLAER VKSGTPPRQGSIT*SPQANEQSVpTPQRR	14 19 18	*Insulin-activated* *Insulin-activated* (s) CAM Kinase GTPPRQGSITSPQAN *Insulin-activated* S854; *Insulin-activated*
splicing factor, arginine/serine-rich 2, (gi|4759172)	KRPQpSPpSPR	17	SR protein-specific kinases (SRPKs) enhances its interaction with the U1 snRNP-specific 70K protein *Insulin-activated*
Splicing factor, arginine/serine-rich 7 (gi|3929380)	SGpSIKGSR	31	RS domain S183 (s) Akt LRRSRSGSIKGSRYF *Insulin-activated*
splicing factor, arginine/serine-rich 9 (gi|4506903)	GSPHYFpSPFRPY	19	RS domain
splicing factor YT521-B (gi|21166355)	RAKpSPpTPDGSER	20	*Insulin-activated*
heterogeneous nuclear ribonucleoprotein K isoform b (gi|14165435)	DYDDMpSPR	17	
translation initiation factor IF2 (gi|15451892)	VEMY*SGpSDDDDDFNKLPK VEMpYpSGSDDDDDFNKLPK SRINpSpSGEpSGDEpSDEFLQSR	21 14 50	 *Insulin-activated*
eukaryotic translation initiation factor 3, subunit 8 (gi|4503525)	RGGYRQQQpSQTAY	27	*Insulin-activated*
eukaryotic translation initiation factor 4B (gi|4503533)	SRTGpSESpSQTGTSTTSSR	53	S422 (S) Akt RERSRTGSESSQTGT T420 (S) Akt QERERSRTGSESSQT
eukaryotic translation initiation factor 4E (gi|4503535)	SGpSTTKNRFVV	19	S209; PKC increases the ability of the protein to bind to mRNA caps and to form the EIF4F complex *Insulin-activated*
tryptophanyl-tRNA synthetase (gi|4759316)	KLpSFDFQ	30	
ribosomal protein P2 (gi|4506671)	KEEpSEEpSDDDMGFGLFD	43	S102;S105; GRK2; regulates activity
ribosomal protein S6 (gi|17158044)	RLpSpSLRA*STSKSESSQK RLSpSLRASTSKpSE*SpSQK	21 18	S235;S236 Phosphorylation involved in cell growth; *Insulin-activated* S236;S244;S247; *Insulin-activated*

	RLpS*SLRA*STSKSESpSQK	17	S240; *Insulin-activated*
ribosomal protein S6 kinase (gi|20149547)	KLPpSTTL	21	*Insulin-activated*
DEAD (Asp-Glu-Ala-Asp) box polypeptide 21 (gi|13787209)	KKEEPSQNDIpSPK	23	
DEAD (Asp-Glu-Ala-Asp) box polypeptide 23 (gi|37542960)	KDHDApSPSKEER	21	
minichromosome maintenance protein 2 (gi|33356547)	RGNDPLTpSSPGR RRGNDPLTSpSPGR GNDPLTpSpSPGRSSR	23 16 15	mitosis-specific protein kinases
nucleolin (gi|4885511)	AIRLELQGPRGpSPNAR TVpTPAKAVpTTPGKK	20 26	Cdc2 kinase in mitosis or casein kinase II S562 (s) Cdc2 ELQGPRG**S**PNARSQP *Insulin-activated* *Insulin-activated*
thymopoietin (gi|4507555)	GPPDFpS*SDEEREPTPVLGSGAAAAGR	38	
apoptotic chromatin condensation inducer in the nucleus (gi|7662238)	SKpSPpSPPRLTEDR RLpSQPESAEKHVTQR RLpSQPEpSAEKHVTQR	20 23 13	
chromodomain helicase DNA binding protein 1 (gi|4557447)	QIDpSpSEEDDDEEDYDNDKR	38	*Insulin-activated*
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c1 (gi|21237802)	KH*SPSPPPPpTPTESR KHpSPpSPPPPTPTESR	21 18	
chromobox homolog 3; heterochromatin protein HP1 gamma (gi|15082258)	RKpSLpSDSESDDSKSK	29	PIM1; may be responsible for some of the alterations in chromatin organization and nuclear structure which occur at various times during the cell cycle S93 (s) PKA KDGTKRK**S**LSDSESD S93 (s) PKC ε KDGTKRK**S**LSDSESD

SMC5 protein (gi|24850456)	KpTSpTPSPQPSK	15	*Insulin-activated*
high mobility group AT-hook 1 isoform b (gi|4504433)	KLEKEEEEGISQEpSpSEEEQ GRPKKLEKEEEEGIpSQE*S*SEEEQ	46 42	CK2 alters its conformation and modulates its DNA binding properties; a subsequent phosphorylation by Cdc2 kinase changes the organization of the protein-DNA complex; consecutive phosphorylation by MAP kinase, which results in a dramatic change in cHMGA conformation, has no direct effect on the complex; attenuates binding affinity and reduces the extent of contacts between the DNA and protein S98 (s) ATM kinase KEEEEGI**S**QESSEEE S98 (s) DNA pk KEEEEGI**S**QESSEEE
HIRA interacting protein 3 (gi|21396500)	KQAREEpSEEpSEAEPVQR	24	S196 (s) casein kinase 2 RKQAREE**S**EESEAEP *Insulin-activated*
meiotic recombination 11 homolog A isoform 2 (gi|24234690)	GVDFEpS*SEDDDDDPFMNTSSLRR	48	*Insulin-activated*
PDGFA associated protein 1 (gi|7657441)	KSLDpSDEpSEDEEDDYQQKR	44	S63 (s) casein kinase 2 KSLDSDE**S**EDEEDDY
cell division cycle 2 protein isoform 1 (gi|4502709)	IGEGpTYGVVYKGR IGEGpTpYGVVYKGR	48 35	T14; Inactivates the enzyme before mitosis T14;Y15; Inactivates the enzyme before mitosis Y15: a key role in the radiation-induced G(2) delay (s) Lck kinase EKIGEGT**Y**GVVYKGR
hepatoma-derived growth factor (gi|4758516)	RAGDLLEDpSPKRPK	33	
neurofibromin (gi|4557793)	SAGpSFKR	35	cAMP-dependent protein kinase? S2829 (s) PKC αβγ QKQRSAG**S**FKRNSIK *Insulin-activated*
CGI-48 protein (gi|7705765)	VQEHED*SGDpSEVENEAK	53	
tumor protein p53 (gi|8400738)	HKKLMFKTEGPDpSD	27	S392; Casein kinase II; binding to 5.8 rRNA
sequestosome 1 (gi|4505571)	SRLpTPVpSPESSSTEEK	23	autophosphorylation or by MBP kinase?
transformer-2 alpha (gi|9558733)	YRRRpSPpSPYYSR	15	RS2 domain S260 (s) PKA DYRYRRR**S**PSPYYSR (s) Akt DYRYRRR**S**PSPYYSR *Insulin-activated*
death-associated protein (gi|4758120)	pSSPPEGKLETK	19	*Insulin-activated*

MLL septin-like fusion (gi|5729933)	HVDSLSQRpSPK	17	
nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate (gi|12232387)	SGKNpSQEDpSEDSEDKDVK SGKNSQEDpSEDpSEDKDVK	26 21	S54 (s) DNA PK KRRSGKNSQEDSEDS S58 (s) Casein kinase 2 GKNSQEDSEDSEDKD
Nuclear ubiquitous casein and cyclin-dependent kinases substrate (gi|13631947)	LKATVpTP*SPVKGKGK	17	CPK1 and casein kinase
protein kinase D2 (gi|19923468)	RLpSpSTSLASGHSVR	40	PKC alpha, PKC epsilon, and PKC eta? S197 (s) Akt GARKRRLSSTSLASG
protein kinase, AMP-activated, alpha 1 catalytic subunit (gi| 40254831)	SGpSVSNYR	32	S487; AMPK kinase *Insulin-activated*
AP2 associated kinase 1 (gi|29570780)	VGSLpTPPS*SPKTQR	25	*Insulin-activated*
tyrosine kinase 2 (gi|31543838)	GARASpPR	17	Protein kinase Cε? *Insulin-activated*
tight junction protein 2 (zona occludens 2) (gi|4759342)	AYpSPEYR GRpSIDQDYER	18 30	Protein kinase C? S244 (s) Akt RDRSRGRSIDQDYER
Opioid binding protein/cell adhesion molecule precursor (gi|37552111)	LTWWEEHQpSPK	15	
peptidyl-prolyl isomerase G (cyclophilin G) (gi|4758106)	KFDHEpSpSPG*TDEDKSG	16	*Insulin-activated*
retinoblastoma-associated factor 600 (gi|24416002)	HApSTpSPSPADKAK	19	*Insulin-activated*
heat shock 105kD (gi|5729879)	IEpSPKLER	28	
spectrin, beta, non-erythrocytic 1 isoform 1 (gi|4507195)	TSSKES*SPIPpSPTSDRKAK	26	S2165 (s) GSK-3K RTSSKESSPIPSPTS *Insulin-activated*
periphilin 1 (gi|31377605)	SYpSPER	16	*Insulin-activated*
ATP-binding cassette, subfamily F, member 1 (gi|10947135)	LKKLpSVPpTpSDEEDEVPAPKPR	28	*Insulin-activated*

up-regulated by BCG-CWS (gi|8272376)	EKVpTGRK	23	*Insulin-activated*
Histidine-rich membrane protein Ke4 (gi|12643344)	EKQpSpSEEEEKETR	25	S276 (s) casein kinase 2 STKEKQSSEEEEKET
Calnexin (gi|10716563)	AEEDEILNRpSPR	18	S563; Protein kinase C?
DnaJ (Hsp40) homolog, subfamily C, member 1 (gi|21361912)	QKDFDIAEQNEpSpSDEESLRKER	15	*Insulin-activated*
dynein, cytoplasmic, heavy polypeptide 2 (gi|37540695)	pSVD*SLKFVA*SWK	16	
LISCH protein (gi|33620723)	NLAL*SREpSLVV	24	

Table 4-2. Phosphopeptides without confirmed phosphorylation sites

* represents that phosphorylation site was not confirmed from raw data due to insufficient MS/MS spetrrum.
(s) represents predicted phosphorylation site and kinases from data search by Scansite (scansite.mit.edu)

Protein	Phosphopeptides	Score	Comments
eukaryotic translation initiation factor 4B (gi|4503533)	SRTGSE*S*SQTGTSTTSSR	77	S422 (s) Akt RERSRTGSESSQTGT
cathepsin L2 (gi|3087790)	KGYV*TPVKNQK	18	
p300/CBP-associated factor (gi|6382076)	*YE*TTQVFGR	17	
tau tubulin kinase 2 (gi|28466991)	QFK*SFLGDL*S*SA*SDKLLEEK	17	cAMP-PK?
pericentrin B (gi|22035674)	RA*TAHTRVPGAHPQPR	15	
zinc finger protein 341 (gi|18158463)	AHIL*SH*SGMKLHK	15	
translokin (gi|7661900)	R*SP*SKP*TLAYPE*SN*SR	15	*Insulin-activated*
myosin IXB (gi|33356170)	Y*TGMLE*TVRIRRSGYSAK	17	
nebulin (gi|4758794)	AVTD*TVSDVK*YK	18	
ADAMTS-20 protein (gi|28316227)	KKRLI*S*YPR	15	
chibby (gi|7656942)	K*SA*SL*SNLHSLDRSTR	16	
oligophrenin 1 (gi|37546035)	SG*SQGQPQ*SR*SGSQR	15	
galactosidase, alpha (gi|4504009)	QEIGGPR*S*Y*TIAVA*SLGK	14	
l(3)mbt-like isoform I (gi|21536479)	KKNL*SGF*SPR	14	
FUS interacting protein (serine-arginine rich) 1 isoform 2 (gi|16905517)	*SA*SHTKTRGT*SKTD*SK	16	
Trp-related protein 4 truncated variant beta (gi|6665592)	*SD*SEEEVAR	17	
Tankyrase 1-binding protein of 182 kDa (gi|20270212)	ASRVP*S(SDEEVVEEPQSR		
nucleolin (gi|4885511)	KVVVSP*TKKVAVATPAK	32	
SWI/SNF-related matrix-associated actin-	KR*SP*SPSPTPEAK	27	

dependent regulator of chromatin c2 isoform a (gi|21237805)			
DNA topoisomerase II, beta isozyme (gi|19913408)	KA*SG*SENEGDYNPGRK	30	
transcriptional coactivator p75 (gi|4050036)	TGVT*ST*SDSEEEGDDQEGEKKR	27	
chromobox homolog 3 (gi|15082258)	RK*SL*SDSESDDSK	27	
adenylyl cyclase-associated protein (gi|5453595)	SGPKPFSAPKPQ*T*SPSPK	21	
similar to vacuolar protein sorting 35 (gi|37541828)	A*SGPVHGEVEER	18	
TP binding protein associated with cell differentiation (gi|18104959)	GKKYD*SD*SDDD	19	
protein kinase, lysine deficient 1 (gi|12711660)	DVDDGSG*SPH*SPHQLSSK	24	
calcium-binding tyrosine phosphorylation-regulated protein isoform a (gi|24797108)	ENEQ*SPR	18	
structure specific recognition protein 1 (gi|4507241)	SKEFV*S*SDE*S*SSGENKSK	18	

Phosphopeptides regulated during early apoptosis induced by tumor necrosis factor-α

* represents that phosphorylation site was not confirmed due to insufficient MS/MS spetrrum.
(s) represents predicted phosphorylation site and kinases from data search by Scansite (scansite.mit.edu)

Table 4-3. Phosphopeptides detected only at 0 hour

Protein (accession No.)	Phosphopeptide	Score	Comments
Ser/Arg-related nuclear matrix protein (gi|5032119)	SRVpSVpSPGR	24	
splicing coactivator subunit SRm300 (gi|19923466)	*SR*SPpSSPELNNK	27	
keratin 19 (gi|24234699)	GVpSVSSAR	23	
epithelial protein lost in neoplasm beta (gi|7705373)	SEVQQPVHPKPLpSPDSR	16	S365 (s) GSK 3K PKPLSPDSRASSLSE
CDC2-related protein kinase 7 (gi|7706549)	E*SRpSSK	18	
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c1 (gi|21237802)	KHpSPpSPPPPTPTESR	33	
cingulin (gi|16262452)	SH*SQApSLAGPGPVDPSNR	31	
transcription elongation factor A (SII), 1 (gi|5803191)	KKEPAITSQNpSPEAR	23	
plectin 1, intermediate filament binding protein 500kDa (gi|4505877)	RApSFAEK	22	P34 cdc2 kinase?
thyroid hormone receptor-associated protein, 150 kDa subunit (gi|4827040)	IDIpSPSTFRK	21	
dyskerin (gi|4503337)	KRE*SEpSEpSDETPPAAPQLIK	24	S452 (s) casein kinase 2 KRKRESESESDETPP
MLL septin-like fusion (gi|5729933)	pSFEVEEVETPNSTPPR	45	
TGFB1-induced anti-apoptotic factor 1 isoform 1 (gi|28416946)	AApSDDG*SLKSSSPT*SYWK	19	
Anillin (gi|31657094)	TQpSLPVTEKVTENQIPAK	28	
Restin (gi|4506751)	TASESISNLpSEAG*SIKKGER	25	S204 (s) PKC αβγ SNLSEAGSIKKGERE FKBP12-rapamycin-associated protein (FRAP, also called mTOR/RAFT)

damage-specific DNA binding protein 2 (48kD) (gi|4557515)	SRpSPLELEPEAK	15	
CASP8 associated protein 2 (gi|6912288)	KKAPPVpTKDPpSpSLK	15	
H1 histone family, member 5 (gi|4885381)	KATKpSPAKPK	19	
progestin induced protein (gi|15147337)	RSpSLSR	20	S287 (s) PKCε YPSFRRSSLSRLGSS
tankyrase 1-binding protein of 182 kDa (gi|20270212)	WLDDLLApSPPPSGGGAR	26	
G protein-coupled receptor kinase-interactor 1; GIT1 protein (gi|7661712)	HGSGAD*SDpYENTQSGDPLLGLEGKR	23	
androgen-induced prostate proliferative shutoff associated protein (gi|7657269)	AEpSPESSAIESTQ*STPQKGR	19	
KARP-1-binding protein (gi|7662142)	LGpSLSAR*SDSEATISR	32	S1062 (s) Akt PRRTRLGSLSARSDS

Table 4-4. Phosphopeptides detected only at 4 hour

Protein (accession No.)	Phosphopeptide	score	comments
splicing coactivator subunit SRm300 (gi|19923466)	SGAGSpSPETK AR*SRpTPPSAPSQSR pSSTPPGESYFGVSSLQLK ELpSNSPLRENSFGpSPLEFR	32 20 36 27	
Ser/Arg-related nuclear matrix protein (gi|5032119)	RRpSPpSPPPTR RQpSPpSPSTRPIR	16 17	S547 (s) PKA PRGRRRR**S**P**S**PPPTR S549 (s) Akt GRRRR**S**P**S**PPPTRRR S711 (s) PKA SSPQRRQ**S**P**S**PSTRP
chromodomain helicase DNA binding protein 4 (gi|4557453)	MSQPGpSPSPK	37	S1535 (s) Cdc2 kinase KKMSQPG**S**PSPKTPT
androgen-induced prostate proliferative shutoff associated protein (gi|7657269)	GRPSKpTP*SPSQPK TPpSPSQPK	19 18	S1379 (s) GSK3 kinase QKGRGRPSKTP**S**PSQ
polypyrimidine tract-binding protein 1 isoform a (gi|4506243)	TDpSSPNQAR	30	
SNF2 histone linker PHD RING helicase (gi|27436873)	KQAVGpSPR	22	S530 (s) Cdk 5 TKKQAVG**S**PRKIQKE
insulin-like growth factor 2 (gi|4504609)	VpSRR*SR	18	
cingulin (gi|16262452)	SKpSLDSR	17	S208 (s) Cam kinase EQRKRSK**S**LDSRLPR Akt EQRKRSK**S**LDSRLPR
cathepsin C isoform a preproprotein (gi|4503141)	RpSGGHSR	16	
zinc finger protein 38 (gi|27544931)	ISpS*SGTAK	15	
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a5 (gi|21071058)	TPTpSPLK	41	
splicing factor, arginine/serine-rich 9 (gi|4506903)	STpSYGYSR	26	
DNA directed RNA polymerase II polypeptide A (gi|4505939)	YSPTpSPK	19	S1839 (s) p38 MAPK SPSYSPT**S**PKYTPTS S1864 (s) P38 MAPK SPKYSPT**S**PKYSPTS S1871 (s) p38 MAPK SPKYSPT**S**PKYSPTS S1920 (s) p38 MAPK SPTYSPT**S**PKYSPTS
similar to 60S ribosomal protein L32 (gi|37552604)	*TKHMLPpSAFRK	18	
mitochondrial transcription	QpSL*SLGQTSISK	18	

termination factor precursor (gi|5902010)			
titin isoform novex-3 (gi|20143916)	SGMAESFAAL*TLpT	19	
isoleucine-tRNA synthetase (gi|4504555)	GAFKAVMTpSIK	16	
3-phosphoinositide dependent protein kinase-1 (gi|4505695)	ANpSFVGTAQYVSPELLTEK	49	S241; known autophosphorylation
plakophilin 3 (gi|6005830)	AGGLDWPEATEVpSPSR ADYDTLpSLR*SLR	23 22	
catenin (cadherin-associated protein), alpha 1, 102kDa (gi|4503127)	TPEELDDpSDFETEDFDVR	36	
signal transducer and activator of transcription 1 isoform alpha (STAT 1) (gi|6274552)	LQTpTDNLLPMSPEEFDEVSR	17	
microfilament and actin filament cross-linker protein isoform a (gi|33188445)	QpTVEAY*SAAVQ*SQLQWMK (?)	17	
SMART/HDAC1 associated repressor protein (gi|14790190)	HG*SFHEDEDPIGpSPR	25	
integrin, beta 4 (gi|21361207)	MDFAFPGpSTNpSLHR	23	
ralA binding protein 1 (gi|5803145)	TPpSSEEIpSPTKFPGLYR	19	
eukaryotic translation initiation factor 3, subunit 8, 110kDa (gi|4503525)	QPLLLpSEDEEDTKR	17	
CDC-like kinase 3 isoform hclk3/152 (gi|4557479)	YRpSPEPDPYLSYR	17	
retinoblastoma 1 (gi|4506435)	ISEGLPpTP*TKMTPR	17	
glutamyl-prolyl tRNA synthetase (gi|4758294)	EYIPGQPPLSQSSDSpSPTR	16	
zinc finger protein 36, C3H type, homolog (gi|4507961)	RLPIFNRIpSVSE	14	
similar to succinate dehydrogenase flavoprotein subunit (gi|29734141)	TLNEADCApTVPPAIR	19	
RNA binding motif protein, X chromosome (gi|4504451)	GLPPpSMER	28	

Table 4-5. Phosphopeptides detected only at 8 hour

Protein (accession No.)	Phosphopeptide	score	comments
thyroid hormone receptor interactor 12 (gi|10863903)	AQTAPTKTpSPR	21	
anillin, actin binding protein (gi|31657094)	ATpSPVKSTTSITDAK	42	
HIV TAT specific factor 1 (gi|21361437)	HFpSEHPSTSK	21	*Insulin-activated*
splicing factor, arginine/serine-rich 2, interacting protein (gi|4759172)	DSpSPGEK	22	
death-associated protein (gi|4758120)	SpSPPEGKLETK	21	
amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein (gi|26665877)	KESKEpTNEK	20	
maternal antigen that embryos require (gi|32481211)	LKSEVVpSPR	17	
nucleolin (gi|4885511)	TVpTPAKAVpTTPGKK	16	PKC zeta?
golgi apparatus protein 1 (gi|6912390)	KVDVVICLSTpTVR	34	
anaphase-promoting complex subunit 4 (gi|7019329)	KpSLHFVK	18	
F-box only protein 3 isoform 1 (gi|15812186)	RLSQLpS*SHDPLWRR	18	
protocadherin beta 3 precursor (gi|9256614)	KpTFQLNPI*TGDMQLVK	18	

Table 4-6. Phosphopeptides detected at 0 and 4 hour

Protein (accession No.)	Phosphopeptide	score	comments
splicing coactivator subunit SRm300 (gi|19923466)	GD*SRpSPSHKR SGpSpSPEVKDKPR SGMpSPEQSR GKR*SLpTR*SPPAIR GEFSApSPMLK R*SSRSpSPELTR	14 21/16 21 15 24 15/29	 T4: S*SRSpSPELTR
scaffold attachment factor B (gi|21264343)	DSEpSHSR	23/60	
Ser/Arg-related nuclear matrix protein (gi|5032119)	RR*SPpSPAPPPR RYpSPpSPPPK RLpSPSApSPPR RH*SPpSPRPR	17/20 20 27/34 15	
apoptosis inhibitor 5 (gi|5729730)	ASEDTTSGpSPPKK ASEDTTpSGSPPKK	20 38	To T4
thyroid hormone receptor-associated protein, 150 kDa subunit (gi|4827040)	*SSSPPPR SSpSPPPR	22 33	To T4
glycogen phosphorylase (gi|5032009)	KQIpSVR	24/31	S14; known by PHK
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c2 isoform a (gi|21237805)	KRpSPpSPSPTPEAK	29/32	
BCL2-associated athanogene 2 (gi|4757834)	SSpSMADR	19/28	
baculoviral IAP repeat-containing 6 (gi|10442822)	EYSARV*SV*TTNTTDSVSDEEKV*SGGK EpYpSAR	15 20	
X-ray repair cross complementing protein 3 (gi|4885659)	KAKLKpSVK	16	
retinoic acid-regulated nuclear matrix-associated protein (gi|7705576)	QTPpSKPK	16	
epidermal growth factor receptor pathway substrate 8-like protein 1 isoform a (gi|21264608)	DNVpTPR DRpSPAAEpTPPLQR	15 15/34	
nuclear receptor coactivator 5	EGpSYDR	21/31	

(gi|15147335)			
SHB (Src homology 2 domain containing) adaptor protein B (gi|4506935)	RQpSpSP*SP*SR RQ*SpSP*SP*SR	16 15	To T4
LIM domain only 7 (gi|33598968)	RGEpSLDNLDSPR	15/21	
IAP-associated factor VIAF1 (gi|13129044)	RDpSDpSEGD	21/36	
keratin 20 (gi|27894337)	LGTTPpSVYGGAGGR LGTpTPSVYGGAGGR	3935 25/27	
trinucleotide repeat containing 15 (gi|32698708)	SQpSWEERGDR	16/20	
mitogen-activated protein kinase 14 isoform 1; p38 MAPK (gi|4503069)	HTDDEMpTGpYVATR	31/35	T180 Y182; known phosphorylation
lamin B1 (gi|5031877)	LKLpSP*SPSSR	29/32	
bromodomain adjacent to zinc finger domain, 1A isoform a (gi|32967603)	LpSSSFSSR	32	
desmoplakin (gi|4758200)	AEpSGPDLR	19/27	S23; known, protein kinase A, PKC?
similar to CGI-148 protein (gi|21945058)	LSpSPPLR	17/24	
splicing factor 3b, subunit 1, 155kDa (gi|6912654)	WDEpTPGR	27/22	
ladinin 1 (gi|20070205)	SApSMKLPDNTVK	17	S394 (s) PKC αβγ TTLTRSASMKLPDNT
stathmin 1 (gi|5031851)	ASGQAFELILpSPR	26/30	S24; known by CDK1 AND MAPK
hepatocellular carcinoma-associated antigen 59 (gi|7706557)	RGDpSEpSEEDEQDSEEVR	42/38	S15 (s) clk kinase FRRRRGD**S**ESEEDEQ S17 (s) casein kinase 2 RRRGDSE**S**EEDEQDS
cortactin isoform a (gi|20357552)	LPSpSPVYEDAASFK	17/55	
sperm associated antigen 9 isoform 2 (gi|27436922)	SApSQSSLDKLDQELKEQQK	32	
catenin (cadherin-associated protein), delta 1 (gi|10835010)	GSLApSLD*SLR	27/38	
epiplakin 1 (gi|13876386)	QVpSASELHTSGILGPETLR	30/53	
similar to Dnajc5 protein (gi|37552868)	SLpSTSGESLYHVLGLDK	27/42	S8 (s) CAM kinase MADQRQR**S**LSTSGES
catenin (cadherin-associated protein), beta 1, 88kDa (gi|4503131)	RLpSVELTSSLFR	39	S675 (s) CAM kinase 2 QDYKKRL**S**VELTSSL PKA QDYKKRL**S**VELTSSL
tight junction protein 2 (gi|4759342)	SR*SWEDSPER SWEDpSPER	15 21	To S170 (s) Akt SHGGRSR**S**WEDSPER T4
tryptophanyl-tRNA synthetase	KLpSFDFQ	25	

(gi|4759316)			
ubiquitin specific protease 8 (gi|4827054)	*SYSSPDITQAIQEEEKR SYpSSPDITQAIQEEEKR	20 25	To 14-3-3 mode I (s) s718 SKLKRSYSSPDITQA T4
butyrate response factor 2 (EGF-response factor 2) (gi|15812178)	RLPIFSRLpSISDD	21	
scinderin; adseverin (gi|14916473)	LpYMVpSDA*SG*SMR	21	
microtubule-associated protein 4 isoform 1 (gi|4505099)	DMEpSPTKLDVTLAK	17/20	MAPK? PKC?
programmed cell death 5 (gi|4759224)	VMDpSDEDDDY	20	
nucleolin (gi|4885511)	KVVVpSPTKK KVVVSP*TKK	46 17	
NICE-4 protein (gi|7661942)	RYPSpSI*SSSPQKDLTQAK	22/20	
MLL septin-like fusion (gi|5729933)	HVDSLSQRpSPK	26	
H2A histone family, member X histone (gi|4504253)	KpTSA*TVGPK KpTSApTVGPK	25 23	To T4
ariadne homolog 2 (gi|5453557)	ADpSYDR	21	
brain-enriched guanylate kinase-associated protein (gi|13124765)	KDpSL*TK	20	
polymerase (RNA) III (DNA directed) (62kD) (gi|21359969)	RRpSpSDEDAAGEPK	17/17	
chromosome 14 open reading frame 118 (gi|8923619)	FKpSAKKQR	17/17	
similar to PBK1 protein (gi|37541364)	FFTpTPSK	21	
endosulfine alpha (gi|4758272)	YFDpSGDYNMAK	45/39	
5'-3' exoribonuclease 2 (gi|18860916)	KAED*SDpSEPEPEDNVR	25/23	
kinesin family member 1B (gi|14349301)	SGLpSLEELR *SGLSLEELR	30 31	To T4
keratin 19 (gi|24234699)	FGPGVAFRAPpSIHGGSGGR APpSIHGGSGGR	28 18	
hepatocellular carcinoma susceptibility protein (gi|20986486)	VMMP*SPASpSMFR	27	
TBC1 domain family, member 4 (gi|7662198)	GRLGpSVDSFER	23/25	S588 (s) Akt RMRGRLGSVDSFERS
lethal giant larvae homolog 2 (gi|4758680)	ARN*SGTQpSDGEEKQPGLVMER	23	
jumonji domain containing 1	NLVGpSEVK	18	

(gi|20357522)			
solute carrier family 9, isoform 3 regulatory factor 1 (gi|4759140)	EALAEAALEpSPRPALVR	18	
protein tyrosine phosphatase, non-receptor type 12 (gi|18375652)	NLpSFEIK	18	S435 (s) CAM kinase KKLERNL**S**FEIKKVP PKC ζ KKLERNL**S**FEIKKVP
transient receptor potential 4 (gi|7706747)	E*SSNSADpSDEK	17	
SH3-domain kinase binding protein 1 (gi|13994242)	ANpSPSLFGTEGKPK	20/20	
scaffold attachment factor B (gi|21264343)	SVVpSFDKVKEPR	18/21	

Table 4-7. Phosphopeptides detected at 4 and 8 hour

Protein (accession No.)	Phosphopeptide	score	comments
microtubule-associated protein 7 (gi|4505101)	AVpSPSNPK	25	
thyroid hormone receptor interactor 12 (gi|10863903)	SApSPDYNR	19/27	
RNA helicase family (gi|24307917)	RWPpTMTYR	14	
heat shock 105kD (gi|5729879)	IEpSPKLER	23/15	
proteasome alpha 3 subunit isoform 1 (gi|4506183)	EpSLKEEDESDDDNM	20/25	T8 (S9?)
H2A histone family, member X; H2AX histone (gi|4504253)	KApTQApSQEY	23	
elongin A (gi|4507389)	*SYSPDHR	18/27	T8: SYpSPDHRQK
protein kinase, interferon-inducible double stranded RNA dependent (gi|4506103)	EKpTLLQK	15/19	

Chapter 5. CONCLUSIONS AND FUTURE STUDIES

5.1. CONCLUSIONS

5.1.1. S-nitrosation regulates the activation of endogenous procaspase-9 in HT-29 cells

Using a biotin labeling method combined with immunoprecipitation, we were able to visualize S-nitrosation of endogenous procaspase-9 in the HT-29 cell line. We suggest that nitric oxide-mediated signals protect cells from apoptosis under normal conditions, via S-nitrosation of procaspase-9, which then is removed during the apoptotic process induced by TNF-α as outlined in Figure 2-7. S-nitrosation could be a major negative regulatory mechanism to explain the role of nitric oxide in protecting cells from apoptosis. On the other hand, denitrosation could be one of apoptotic events induced by TNF-α to speed up the cleavage of procaspase-9.

5.1.2. The inhibitory effects of insulin on the activation of procaspase-9 via X-chromosome linked Inhibitor of Apoptosis Protein (XIAP)

We suggest that an interaction between XIAP and procaspase-9 is one of the regulatory systems by which insulin decreases the TNF-α-induced cleavage of procaspase-9 and the subsequent apoptosis. Results in this thesis, along with those of Janes et al. (Chapter 3 ref. 82), support an anti-apoptotic mechanism in which insulin acts through the PI-3K/Akt pathway. Further, a phosphorylation event (s) on an Akt substrate (s) may prevent release of XIAP from procaspase-9.

5.1.3. Detection of phosphoproteins potentially regulated during apoptosis induced by tumor necrosis factor-α

Diversity of potential regulatory mechanisms led us to investigate alteration of phosphorylation in apoptotic HT-29 cells at global level. ~200 phosphopeptides were identified from insulin-treated cells. The list includes signaling proteins such as p53, CDC 2 isoform 1, PDGFA associated protein-1, Protein kinase D2, PKA alpha 1 catalytic subunit, and AP2 associated kinase-1. In detecting alterations of phosphopeptides during early apoptosis, unknown phosphorylation sites from our data may add potential regulations to known regulatory mechanisms of both anti- and pro-apoptotic proteins. Further isolation of a group of interesting peptides or additional fractionation will simplify samples and, therefore, enable us to identify and quantitate a larger number of low-abundance molecules involved in signaling pathways.

5.2. FUTURE STUDIES

5.2.1. S-nitrosation of proteins

5.2.1.1. Development of analytical methods to identify S-nitrosated peptides

We have been developing an analytical method to enrich S-nitrosated peptides. Although it was successful to analyze a single peptide, application of the method to exogenously nitrosated proteins demonstrated difficulties most likely due to the limit of detection resulting from low-efficient nitrosating reaction or loss of samples during the procedure. The procedure, therefore, needs to be improved by decreasing the number of steps to enrich S-nitrosated peptides and enhancing detection sensitivity.

5.2.1.2. Identification of a nitric oxide synthase and other mediators responsible for S-nitrosation

We could not detect responsible nitric oxide synthase by Western blotting. Stamler group reported co-localization of iNOS and procaspase-3, which suggests that a NOS and procaspase-9 may be compartmentalized as well. Whether a NOS is located in mitochondria is not certain, but previous literature supports a hypothesis that mitochondrial NOS is likely a source of S-nitrosation at least for the proteins localized in mitochondria since larger fraction of procaspase-3 is S-nitrosated in mitochondria, yet this modification did not affect localization. Identification of not only a source of S-nitrosation, but also direct S-nitrosating molecules is fundamental question to be answered. Molecules involved in cellular redox systems, such as glutathione, thioredoxin, can be reasonable to investigate for their ability to transnitrosate proteins.

5.2.2. Regulation of the cleavage of procaspase-9 via XIAP

We demonstrated that insulin decreased TNF-α-induced apoptosis, in part, by decreasing the cleavage of an upstream caspase, procaspase-9, via XIAP through PI-3K/Akt pathway. Investigating involvement of phosphorylation in interaction of procaspase-9 and XIAP will be informative to understand regulation of apoptosis by survival factors.

5.2.3. Proteomics approach to investigate regulation of phosphorylation during apoptosis

Phosphoproteomics approach at the global level demonstrated advantage in obtaining information of a number of proteins at one time, but most phosphoproteins identified with high score are high abundance proteins. In order to investigate low-abundance proteins involved in signaling pathways, additional sample preparation procedure such as multi-dimensional chromatography, subcellular fractionation, or immunoprecipitation is necessary. Simplifying samples will help also to obtain quantitative information on low-abundance phosphoproteins in addition to the advantage from improved quantitative techniques such as co-eluting isotope labeling agents.